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Differential Removal of Strontium 85 and Calcium 45 from Rat Skeleton By Peritoneal Lavage. (23238)

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The need for understanding the behavior of strontium in various biological systems, especially in relation to calcium, is emphasized by the possibilities and implications of contamination of the biosphere with strontium 90 (1,2). In general, it has been shown that of the two elements, calcium is preferentially absorbed from the gastrointestinal tract, secreted in milk, and transported to the fetus;

on the other hand, strontium is preferentially excreted in urine, in bile, and into the digestive tract (3-6). Attention has now been directed toward the comparative movement of calcium and strontium between body fluids and bone. The effects produced by physiological processes already mentioned make it difficult to observe in the intact animal any differential behavior directly related to bone. Lengemann (7), using tissue cultures of embryonic chick bones, showed that the Sr^*/Ca^* ratio was 1.08 in newly formed bone as compared with a value of 1 in the substrate, but that the ratio was 1.2 for the Sr^*/Ca^* in sub-

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strate being derived from labeled bone having a ratio of 1. Comar *et al.* (5), from daily ingestion studies of radiostrontium and radiocalcium in rats, showed that the Sr^*/Ca^* of bone was equal to or slightly higher than that of blood when plateau levels in each tissue had been reached.

In the present work, a direct comparison was made of removal of previously deposited calcium 45 and strontium 85 from normal and nephrectomized rats by peritoneal lavage.

Methods. Male rats (Carworth) weighing between 160 and 250 g were used. Normal rats were maintained on stock diet but were given no food the night before the experimental run. Nephrectomized rats were put on a salt deficient diet the night before removal of the kidneys and were maintained on this diet for duration of the experiment. Nephrectomy was done by ventral mid-line approach. Each animal received an intra-peritoneal injection of a solution containing about 50 μC of Ca 45 and 5 μC of Sr 85, both essentially carrier-free. Calcium, phosphate, and Ca 45 analyses were done by standard methods (8) and the Sr 85 was counted in a scintillation well-counter. *Peritoneal Lavage.* The method is a modification of that reported by Talmage and Elliot (9); however, because of its simplicity and possible usefulness it is described in some detail. The catheter was made of glass tubing flattened perpendicularly on the end and containing 3 openings recessed behind the button-like end. About one-quarter inch behind the openings, the tube was ridged for holding the suture thread. The remainder of the glass tube was inserted into rubber tubing controlled by a screw clamp and placed into a graduated cylinder for collection of the peritoneal wash. The incision was made at the anterior end of the inguinal canal, after which the spermatic cord with its accompanying adipose tissue was stretched from the peritoneal cavity and tied off. The catheter was then slipped through the canal into the peritoneal cavity. A suture was stitched through the layers of the inguinal wall and tied around the ridge provided on the catheter thus preventing escape of fluid around the catheter and at the same time holding it far enough into the peritoneal cavity

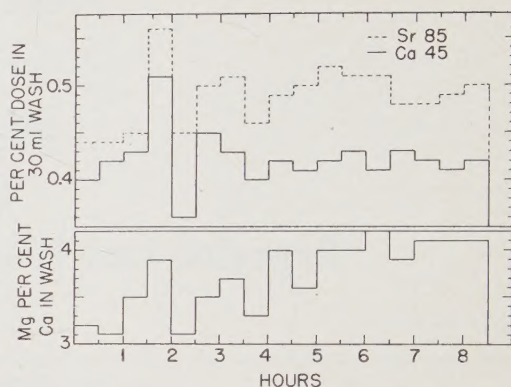


FIG. 1. Removal of Sr 85, Ca 45, and Ca from labeled rat skeleton in peritoneal lavage solutions collected every 30 min.

that the walls of the cavity would not close the openings. The button-like end prevented the intestines from blocking the openings. The testis was either cut off or sewed into the scrotum. The animal was maintained under nembutal anesthesia throughout. By this procedure the wash was normally collected merely by releasing the screw-clamp on the catheter; however, occasionally a slight pressure on the abdomen was needed to drain sufficient fluid. At the beginning of the experiment, 35 ml lavage fluid were placed in the peritoneal cavity either through the same catheter or through a polyethylene catheter placed in the abdomen under the liver. At the end of each equilibration period, 30 ml were removed and the same amount was replaced in the cavity. The lavage fluid consisted of 0.84% NaCl and 1% dextrose. Duration of equilibration periods and length of the experiment varied with experimental needs.

Results. Fig. 1 presents a typical histogram showing removal of strontium 85, calcium 45, and calcium in the lavage solutions collected every 30 minutes during an 8-hour period. This particular animal had been nephrectomized and had received the radioisotopes 24 hours before the start of the lavage procedure. The main features are (a) greater removal of Sr 85 than Ca 45 (as will be noted later these animals had the same amount of Sr 85 and Ca 45 in the bones at start of lavage); (b) general simultaneous changes of Sr 85, Ca 45, and Ca; and (c)

TABLE I. Removal of Ca 45, Sr 85, Ca and P from Rats by Peritoneal Lavage. (8-hr treatment with 30-min. equilibration periods.)

Exp. condition	Normal	Nephrect.	Nephrect.	Normal
Time between isotope dosage and lavage	24 hr	24 hr	48 hr	8 days
No. of animals	6	6	4	2
% of dose in skeleton*				
Ca 45	83 \pm .5	87 \pm 2	91 \pm .5	75
Sr 85	76 \pm 2	87 \pm 2	89 \pm 1	56, 57
% of dose removed*				
Ca 45	3.5 \pm .2	5.9 \pm .5	1.7 \pm .1	.40, .38
Sr 85	3.7 \pm .2	7.5 \pm .6	2.5 \pm .1	.33, .30
% of skeletal burden removed				
Ca 45	4.2	6.7	1.9	.51
Sr 85	4.8	8.7	2.8	.57
Electrolyte removed†				
Ca, mg/ml of wash	.035-.045	.030-.040	.025-.035	
P, mg/ml of wash	.015-.025	.025-.040	.035-.045	

* Mean \pm stand. error of mean.

† Range over 8-hr period of exp.

relatively uniform removal over the 8-hour period.

A study was made of the effect of time of peritoneal equilibration (between 15 minutes and 1 hour) upon rate of electrolyte removal from the body. Removal rate was maximum when 15-minute periods were employed; when 30-minute and 1-hour periods were used removal rates were about 70% and 45%, respectively, of the 15-minute values. For convenience, a 30-minute period was used for most studies.

Table I presents data first on amounts of Ca 45 and Sr 85 retained in the skeleton of normal and nephrectomized rats at 24 hrs, 48 hrs, or 8 days after intraperitoneal injection. The values are given as percentage of dose in the skeleton at start of lavage; they were obtained by analysis of the carcass at the end of experiment, to which values the amounts removed by lavage were added.

Data are then given for the amounts of Ca 45 and Sr 85 removed during 8 hours of peritoneal lavage with 30-minute equilibration periods. The amounts removed are expressed both as percentage of dose and as percentage of skeletal burden at start of lavage. Values are also given to indicate amounts of calcium and phosphorus removed, expressed as range in mg/liter of wash during the 8-hour period.

Table II presents a definition of the term "Strontium-Calcium Observed Ratio (OR)" and gives calculated values for these ratios that permit more accurate estimation of the comparative removal of the 2 isotopes from bone(5). Values are presented for the discrimination in movement of the 2 elements between dose and bone, dose and lavage solution, and between bone and lavage solution.

Discussion. The data in Table I for percentage of dose in skeletons of rats at 24 hours after injection show that nephrectomy

TABLE II. Differential Removal of Ca 45 and Sr 85 from Rats by Peritoneal Lavage.

	Normal	Nephrect.	Nephrect.	Normal
	Time between isotope dosage and lavage			
	24 hr	24 hr	48 hr	8 days
	No. of animals			
	11	6	4	3
OR _{bone-dose} *	.92 \pm .02	.99 \pm .001	.99 \pm .01	.77
OR _{lavage-dose}	1.03 \pm .06	1.30 \pm .05	1.50 \pm .03	.79
OR _{lavage-bone}	1.12	1.31	1.52	1.03

$$* \text{OR}_{\text{bone-dose}} = \frac{\text{Sr}^*/\text{Ca}^* \text{ in bone}}{\text{Sr}^*/\text{Ca}^* \text{ in dose}} (5).$$

had only slight if any effect on calcium retention, but did increase retention of strontium. Even at 48 hours after injection of nephrectomized rats, calcium and strontium retentions were almost equal. This supports earlier work(5,10) and demonstrates that renal discrimination is of major importance in the preferential removal of strontium once it is within the body. The greater retention of Ca 45 than Sr 85 at 8 days after injection into the normal animal reflects primarily the preferential urinary excretion of strontium during this period.

Let us now consider the removal of Ca 45 and Sr 85 by lavage; it can be seen from the 24-hour animals that nephrectomy increased the amounts of Ca 45 and especially of Sr 85 that were removed by lavage. This increase is probably due to loss in competition for electrolytes exerted by the kidney in the intact rat.

It is of interest to note the effect of length of time in the body on availability of radioisotopes for removal by lavage. For example, Sr 85 and Ca 45 in the body for 48 hours were only about one-third as effectively removed as that in the bone for 24 hours; after 8 days, the isotopes were only about one-eighth as available for removal. This behavior is a consequence of normal processes taking place in bone such as accretion, resorption, and redeposition in growth or remodeling, plus possible physical changes(6). It is pointed out that although these data demonstrate that such methods (artificial kidney) can in principle be used to remove radioactive calcium analogues from the body, they are inefficient and would require considerable improvement before being practical. The importance of treatment soon after exposure is also stressed.

The comparative retention in the skeleton as affected by urinary discrimination can also be expressed as an OR (Observed Ratio) value as shown in Table II. The $OR_{\text{bone-dose}}$ near unity for the nephrectomized groups, and the value of 0.77 for the 8-day normal animals again emphasizes the effect of preferential urinary excretion of strontium.

A main objective of this study was to estimate the comparative removal of the 2 isotopes from bone under conditions in which

the kidney discrimination was not in effect. It was thought unlikely that significant physiological discrimination would be caused by any of the processes involved in the movement of calcium and strontium from the circulating fluids to the lavage solution. Accordingly, the discrimination that occurs between bone and peritoneal lavage solution in the nephrectomized animal is attributed to differential removal from bone. Interference resulting from the discrimination due to endogenous excretion into the gastrointestinal tract is probably not serious.

It is noted in Table II that the $OR_{\text{lavage-bone}}$ ratio in the nephrectomized animals was significantly higher than unity (1.3 to 1.5). This is interpreted to mean that Sr 85 was being preferentially released from bone as compared with Ca 45. The $OR_{\text{lavage-bone}}$ values in the normal animals have little meaning because of the masking effects of the urinary discrimination. The value of 1.3 for the 24-hour nephrectomized rats is in agreement with the value of 1.2 found by Lengemann(7) for the Sr/Ca released from bone in tissue culture. It seems, therefore, that the ratio of Sr/Ca released from bone is about 1.3 times the Sr/Ca in bone.

It was of interest to estimate the ratio of Sr/Ca that enters bone. This has been done with the use of the Sr/Ca ratio of 1.3 for the elements leaving bone, in conjunction with other data from the literature. The formula and calculation are shown in Table III. In principle, if one has (a) steady state values for Sr/Ca in bone and blood, (b) the bone accretion and resorption rate for calcium, and (c) the Sr/Ca ratio leaving bone, it is then possible to calculate the Sr/Ca ratio entering bone. Values for (a) have been reported for three groups of rats(5) and Bauer *et al.*(11) have estimated that in the whole tibias of young rats, the calcium accretion was 0.17 mg/hr and calcium loss by resorption was 0.13 mg/hr. When these values are used as shown in Table III, it is calculated that the Sr/Ca ratio entering bone was 1.5, 1.3, and 1.6 times the blood Sr/Ca ratio for the three groups of rats previously described.

Summary. 1) A simple technic has been described for removal of electrolytes from the

TABLE III. Calculation of Ratio of Strontium to Calcium Moving from Blood to Bone of Rats. (D = Sr/Ca ratio in diet.)

Group		1	2	3	Ref.
Sr/Ca in bone at steady state	(B)	.57 D	.45 D	.27 D	(5)
" " blood " " "	(P)	.46 D	.43 D	.21 D	(5)
Accretion rate of calcium	(A)	.17	.17	.17	(11)
Resorption " " "	(R)	.13	.13	.13	(11)
Sr/Ca leaving bone	(L)	1.3	1.3	1.3	
" entering "	(E)	1.5	1.3	1.6	

Note: Equation for E derived as follows:

$$\frac{\text{Sr in bone}}{\text{Ca in bone}} = \frac{\text{Rate of Sr entry} - \text{Rate of Sr leaving}}{\text{Rate of Ca entry} - \text{Rate of Ca leaving}} = \frac{(A)(E)(P) - (R)(L)(B)}{A - R} = B,$$

$$\text{and } E = \frac{B(A - R + RL)}{(A)(P)}.$$

rat by peritoneal lavage. From comparisons of strontium/calcium ratios in lavage solutions and bones of nephrectomized rats it is estimated that strontium was preferentially released from bone by a factor of 1.3 over calcium. 2) It was calculated that strontium preferentially entered bone from blood by a factor of 1.3 to 1.6. The differential removal of the 2 elements from the intact rat was shown to be due mainly to renal function. Eight hours of peritoneal lavage removed about 5% of the Sr* that had been deposited for 24 hours but only about 0.6% of that deposited for 8 days.

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Effects of Testosterone, Sesame Oil, and Castration on Tissue Respiration of Male Rats Exposed to Cold. (23239)

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Addition of testosterone *in vitro* to tissue slices is reported to have either an augment-

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ing(1) or a depressing(2,3) effect on rate of oxygen uptake of tissue slices depending upon concentration. Smith, Kochakian and Fondal(4) report a decrease in rate of oxygen uptake of tissue slices and homogenates of liver and kidney from rabbits, rats and guinea

TABLE I. Oxygen Uptake of Kidney Slices Recorded at 15 Min. Intervals between 30 and 105 Min. Incubation Period.

Incubation time		45	60	75	90	105	S.D. of line	Dev. of 75 min. value
Control	O	13	12	10	11	10	.66	
	C	13	12	12	11	10		
Control— Test. <i>in vitro</i> *	O	16	18	11	17	15	1.75	-4.50†
	C	17	16	—	15	14		
Castrate control	O	20	16	19	20	16	1.66	
	C	18	18	18	18	18		
Castrate control— Test. <i>in vitro</i> *	O	18	20	18	21	15	1.85	- .75
	C	19	18	—	17	16		
Oil—23°C	O	14	15	11	12	10	.78	-2.50†
	C	14	14	—	13	—		
2°C	O	13	13	10	13	10	.53	-3.00†
	C	13	13	—	13	—		
T.O.—23°C	O	15	14	25	38	20	1.47	-5.25†
	O	17	17	14	12	16		
2°C	C	17	17	—	—	17		
<i>Castrated</i>								
Oil—23°C	O	17	16	14	16	14	.54	-1.30‡
	C	17	16	—	15	14		
2°C	O	15	13	11	14	14	.93	-2.70†
	C	14	14	—	13	13		
T.P.—23°C	O	16	15	12	13	15	.84	-2.40‡
	C	15	15	—	14	14		
2°C	O	16	18	12	18	16	-.93	-5.00†
	C	17	17	—	17	17		

* Testosterone added *in vitro* after 1 hr incubation to all groups except control and castrated control.

† Significantly different from calculated line at 1% level.

‡ *Idem*, at 5% level.

O—observed value; C—calculated value.

pigs, when testosterone is added to the incubation medium. Dirscherl and Hauptmann (1) demonstrated a stimulating effect of testosterone in low concentrations in contrast to a depressing effect reported by other investigators.

This experiment was performed to determine the effect of testosterone added *in vitro* upon respiration of tissues from cold exposed rats. Testosterone was added to tissue slices from castrated and intact animals, previously treated with testosterone propionate or sesame oil, and exposed to a low ambient temperature for 50 days.

Methods. Oxygen uptake of approximately 90 samples each of liver, kidney and brain slices from male Sprague-Dawley rats was determined in the Warburg constant volume respirometer. Tissues were obtained from animals treated as follows. Rats were treated daily with either 1 mg testosterone propionate

in 0.1 ml sesame oil, or sesame oil alone for 50 days. Half of the animals were maintained at room temperature and half were kept at $2 \pm 2^\circ\text{C}$ for the same periods and treated similarly. Half of the rats maintained at each temperature were castrated at 30 days and all treatments started at 60 days of age. Rats in cold were kept in individual wire cages without nesting material; those at room temperature were housed 5-10 animals/cage. Food and water were given *ad libitum*. The animals were killed by cervical luxation. Samples of liver, kidney and brain were removed and transferred to cold Krebs-Ringer-Phosphate (K-R-P) solution (pH 7.41) within 2 minutes after death. Tissues were sliced in a Stadie-Riggs microtome, weighed to 0.1 mg and placed in Warburg flasks containing 2 ml of cold K-R-P. Flasks were kept in ice until the flask-manometer systems were transferred to water bath at $38 \pm 0.01^\circ\text{C}$. After

TABLE II. Oxygen Uptake of Brain Slices Recorded at 15 Min. Intervals between 30 and 105 Min. Incubation Period.

Incubation time		45	60	75	90	105	S.D. of line	Dev. of 75 min. value
Control	O	11	7	6	4	4	1.38	
	C	10	9	7	5	4		
Control— Test. <i>in vitro</i> *	O	8	9	4	7	6	.75	-2.50†
	C	9	8	—	6	6		
Castrate control	O	12	8	9	11	5	1.85	
	C	12	11	9	8	7		
Castrate control— Test. <i>in vitro</i> *	O	14	11	11	11	8	1.00	- .25
	C	14	13	—	10	8		
Oil—23°C	O	9	6	2	8	5	.84	-4.40†
	C	9	7	—	6	5		
2°C	O	11	7	7	8	5	1.25	-2.60‡
	C	11	10	—	8	7		
T.P.—23°C	O	11	10	13	24	9	1.41	-5.40†
	C	12	13	5	8	5		
2°C	O	14	12	—	9	7	1.41	-5.40†
	C	14	12	—	9	7		
<i>Castrated</i>								
Oil—23°C	O	14	12	8	10	8	.75	-2.90†
	C	14	13	—	9	7		
2°C	O	11	11	4	9	5	2.62	-6.20‡
	C	12	11	—	9	9		
T.P.—23°C	O	13	10	7	9	9	1.41	-3.50‡
	C	13	12	—	9	7		
2°C	O	12	10	5	11	8	1.85	-4.50‡
	C	13	11	—	8	6		

* Testosterone added *in vitro* after 1 hr incubation to all groups except control and castrated control.

† Significantly different from calculated line at 1% level.

‡ *Idem*, at 5% level.

O—observed value; C—calculated value.

15 minutes equilibration, manometers were read at 15-minute intervals during 2 hours incubation. Testosterone (0.2 ml) was tipped into the reaction chamber from sidearms after one hour. Testosterone was dissolved in dehydrated ethyl alcohol and diluted to 10% immediately before use. Final concentration was 2 mM. Tissues of intact and castrated controls were divided into 2 groups. Testosterone was added *in vitro* to tissues from 1 group of intact and 1 of castrated rats, whereas the remainder of tissues were permitted to respire for 2 hours with no addition of testosterone. Although values were obtained at 15 minute intervals during the entire period, the data presented in Tables I and II are for incubation time between 30 and 105 minutes. This procedure was followed to demonstrate changes immediately after addition of testosterone. A straight line

curve was calculated by the method of "least squares" using the 15 minute values. For this calculation the value for the interval immediately following addition of testosterone (75 minute value) was not incorporated. To ascertain whether testosterone had significantly altered oxygen uptake the deviation of this value (75 minute value) was determined and compared to the deviation of other values on the line.

Results. No significant change in rate of oxygen uptake appeared in liver slices from any of the animals regardless of whether testosterone was added.

Effects of testosterone in vitro on tissue respiration of control rats. The following data are illustrated in Tables I and II and Fig. 1. The control group maintained at room temperature ($23 \pm 2^\circ\text{C}$) and given no treatment either *in vivo* or *in vitro* had a con-

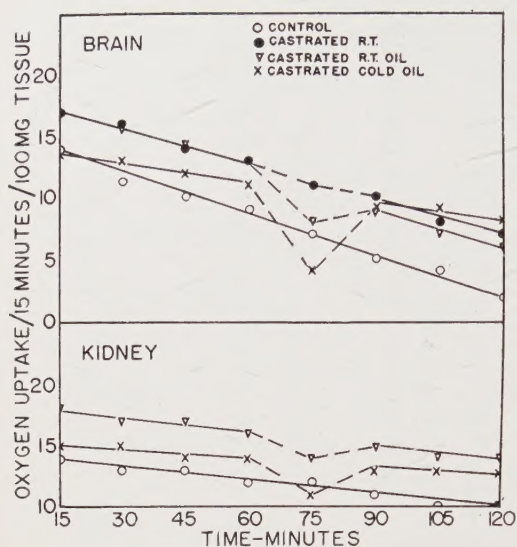


FIG. 1. Curves are calculated as "best fit" lines by the method of least squares; the 75 min. values are observed values. R. T., room temperature; oil, Sesame oil.

stant rate of oxygen uptake with no change in the 75 minute values for either kidney or brain tissue. Oxygen uptake of kidney and brain slices from controls maintained at room temperature and receiving no treatment *in vivo*, but with testosterone added *in vitro* showed a significant decrease in the 75 minute value. The oxygen uptake curves, both calculated and observed, for kidney and brain slices, from castrated rats kept at room temperature with no treatment either *in vivo* or *in vitro*, were not changed at any point. This was to be expected. Tissue slices from castrated rats maintained at room temperature showed no change in rate of oxygen uptake when testosterone was added *in vitro*.

Effects of testosterone propionate and sesame oil in vivo and testosterone in vitro on tissue respiration. Rats at room temperature treated with sesame oil showed a significant difference in rate of oxygen uptake after *in vitro* addition of testosterone to kidney and brain slices. A corresponding group exposed to cold ($2 \pm 2^\circ\text{C}$) also showed a significant decrease in rate of oxygen uptake after *in vitro* addition of testosterone. These decreases were significant at 1 and 5% levels. Intact animals treated *in vivo* with testosterone propionate and housed at room tem-

perature showed a significant increase in rate of oxygen uptake from kidney and brain slices. However, testosterone propionate administered *in vivo* during exposure of rats to cold resulted in a highly significant depression of oxygen uptake in both kidney and brain slices after *in vitro* addition of testosterone. Intact animals treated *in vivo* with sesame oil and kept at room temperature show a significant decrease in rate of oxygen uptake after *in vitro* addition of testosterone. Animals treated in the same way except exposed to cold gave similar results. This applies to kidney and brain slices. Castrated rats treated with testosterone propionate *in vivo* either at room temperature or exposed to cold gave results of similar pattern to groups treated with sesame oil. This applies to both kidney and brain slices.

Discussion. The failure of testosterone to alter oxygen uptake of liver slices was probably due to low concentration of hormone used in these experiments. However, depression of oxygen uptake by kidney and brain slices from intact animals following addition of testosterone *in vitro* is in agreement with previous reports(2,3) as is failure to note any effect on rate of oxygen uptake following *in vitro* addition of testosterone to tissue from castrated male rats(3).

Castration induces a change in response of tissue to testosterone propionate, sesame oil and cold. Treatment *in vivo* with testosterone propionate or sesame oil restores the capacity of tissues to respond to *in vitro* addition of testosterone; however restoration of this response by sesame oil is limited. Tissue from untreated castrated rats did not respond. The decrease in rate of oxygen uptake following addition *in vitro* of testosterone to kidney and brain slices from castrated rats treated with oil and exposed to either room temperature or cold indicates an action for sesame oil similar to that of testosterone propionate. It appears that both sesame oil and testosterone propionate may have sensitized the tissue in such a manner that depression of oxygen uptake resulted after addition of testosterone *in vitro*. Physiological responses to sesame oil have been reported by others. Crafts(5) reports a de-

crease in weight of prostate and seminal vesicles from animals treated with androsterone dissolved in sesame oil or sesame oil alone. Tobin(6) found sesame oil prolonged survival of pregnant rats adrenalectomized after onset of pregnancy. He concluded that under these conditions sesame oil is not an inert vehicle. Our results, while not dealing with the same responses, indicate that sesame oil may be physiologically active(7).

Tissue slices from intact rats treated daily with either testosterone propionate or sesame oil responded to *in vitro* addition of testosterone by a decrease in rate of oxygen uptake with one exception. This increase was found in tissues from intact animals treated with testosterone propionate and maintained at room temperature. No explanation is offered.

Liver metabolism of steroids is complex, therefore, it is probable that the liver can metabolize a greater number of compounds than can other tissues(8) without a significant alteration in respiration. Kochakian (9) has presented evidence that kidney and liver do react differently in the metabolism of androgens.

Our results indicate that treatment with sesame oil *in vivo* restores the capacity of tissues from castrated male rats to respond to testosterone *in vitro*. Exposure to cold enhances this action of sesame oil.

Summary. 1. The effect of addition of testosterone (2mM) *in vitro* to liver, kidney and brain slices from intact and castrated rats treated with testosterone propionate and sesame oil *in vivo* and exposed to cold ($2 \pm 2^\circ\text{C}$) was studied in the Warburg constant

volume respirometer. 2. No significant change was found in rate of oxygen uptake of liver slices with any treatment. These results with liver reflect a more rapid rate of destruction of testosterone. Kidney and brain slices from intact animals show a decrease in rate of oxygen uptake after addition of testosterone *in vitro*. Kidney and brain slices from untreated castrated rats kept at room temperature exhibit no change in rate of oxygen uptake after addition of testosterone *in vitro*. 3. Tissue from castrated rats treated with sesame oil and maintained at room temperature or in cold show a decrease similar to tissues from intact animals following addition of testosterone *in vitro*. 4. All changes were temporary and inhibitory, except in the case of animals treated with testosterone propionate *in vivo* at room temperature.

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Comparative Hemolytic Activity of Mephenesin, Guaiacol Glycerol Ether and Methocarbamol *in vitro* and *in vivo*. (23240)

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Two impediments to intravenous use of mephenesin are its hemolytic effect and the large volumes of solution required (1-6.) Guaiacol glycerol ether [3-(*o*-methoxyphenoxy)-1,2-propanediol] (GG) has comparable muscle relaxant effects, an expectorant action and much weaker hemolytic properties (7-14). Methocarbamol [3-(*o*-methoxyphenoxy)-1,2-propanediol monocarbamate] (AHR-85) has similar properties of extended duration with adequate solubility for parenteral use (15).

This investigation details *in vitro* and *in vivo* comparisons between GG, AHR-85 and mephenesin.

Methods. *In vitro*. Hunter's method (16) for erythrocyte fragility was adapted to variations in drug concentration. Drugs were dissolved in 0.015 M $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer (pH 7.4) in 0.9% NaCl with the following increments and ranges of concentration; mephenesin, 0.02% (0.82 to 0.92%), GG, 0.2% (2.0 to 3.0%) and AHR-85, 0.1% (2.0 to 2.5%). Heparinized, rather than oxalated blood, was used. Measurements were made of oxyhemoglobin in a Beckman spectrophotometer at 540 $\text{m}\mu$ after standing 55 minutes (with intermittent shaking) in a 25°C water bath followed by 5 minutes of centrifugation. Per cent hemolysis at each concentration was calculated by the formula:

$$\% \text{ Hemolysis} = \frac{\text{Opt. density of sample}}{\text{Opt. density of 100\% hemolysis}} \times 100.$$

The concentration of each drug producing 50% hemolysis (HC_{50}) was interpolated visually from a probit of % hemolysis versus log of concentration graph. This probit transformation of % hemolysis values seemed valid since Ponder (17) has shown the log

concentration curves to be essentially sigmoidal except for a small amount of skewness near 100% caused by highly resistant erythrocytes.

Results. *In vitro*. The results obtained are set forth in Table I along with the calculated relative hemolytic activity of GG and AHR-85 compared to mephenesin as 100%. It can be seen that there is a small amount of variability in the concentrations required for the HC_{50} in different individuals, but the ratios between drugs for the same persons' erythrocytes are very uniform. The averages of these values indicate that at equihemolytic concentrations GG is about 31% and AHR-85 is close to 39% as hemolytic compared to mephenesin as a standard.

Methods. *In vivo*. The methods used were the van den Bergh test for total serum bilirubin and the method of Hunter *et al.* (18) for serum hemochromogen. Five unanesthetized dogs were used for 11 experiments using doses of 100 mg/kg of mephenesin and equimolar doses of GG (109 mg/kg) and AHR-85 (133 mg/kg). The drug solutions were injected intravenously at a rate of approximately 15 cc/min. after prior warming to 37°C. The concentrations used were mephenesin—2% (as Tolserol - Squibb, a supersaturated solution), AHR-85 - 3 and 4% (as aqueous solutions, the latter is saturated at 37°C) and GG - 4 and 5% (as aqueous solutions, the latter is saturated at 37°C). Venous blood samples were taken with a dry siliconized syringe and needle for a control, 30 minute, 1 hour and 2 hour samples. Serum bilirubin differences were taken between the control sample and the 30 minute, 1 hour and 2 hour samples for 5 control dogs. Student's *t* value was calculated for the bilirubin changes in drug experiments in comparison to the mean of these control differences and their standard devi-

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TABLE I. Concentrations of Mephenesin, GG and AHR-85 Producing 50% Hemolysis of Human Venous Blood in 1 Hr (HC_{50}) and Relative Activity between Drugs.

Donor	EBT	AMM	WCB	RBP	LAW	JC	Avg
50% hemolytic conc., %							
Mephenesin	.88	.85	.90	.88	.885	.87	.875
	.88	.84		.89			
GG	2.84	2.73	2.93	2.92	2.88	2.82	2.85
AHR-85	2.22	2.16	2.34	2.29	2.26	2.27	2.26
Relative hemolytic activity, % (mephenesin = 100%)							
Mephenesin	31	31	30.7	30.3	30.7	30.8	30.75
GG							
Mephenesin	39.7	39.1	38.5	38.6	39.2	38.3	38.9
AHR-85							

ation at comparable times by the formula:

$$t = \frac{\bar{X}_{\text{Cont.}} - \bar{X}_{\text{Exp.}}}{\text{S.D.} \cdot \bar{X}_{\text{Cont.}}}$$

Results. In vivo. The changes in serum bilirubin from the dogs' own controls are plotted in Fig. 1. Significant differences ($p \leq 0.05$) are indicated by an "s." It may be seen from the graphs that the elevations of serum bilirubin above control values were not large in any instance. Intravenous infusion

of 2% mephenesin caused a rise in serum bilirubin which was in most cases intermediate between that of the stronger and weaker solutions of GG and AHR-85 used.

With the serum hemochromogen test it was found that measureable hemolysis occasionally occurred in control samples. Thus it was not possible in all instances to determine whether the total amount of hemolysis was attributable to the drug alone or was caused in part by mechanical trauma to the blood

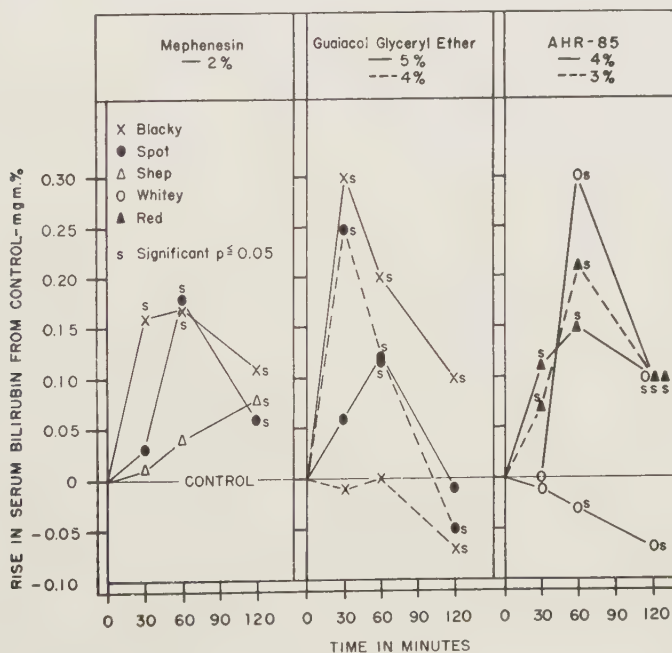


FIG. 1. Changes in serum bilirubin from control values in dogs after rapid intrav. inj. of aqueous solutions of mephenesin, GG and AHR-85. See text for explanation of test of significance.

cells during the handling of the blood. However, in no instance after infusion of a drug did the hemochromogen value exceed 88 mg/100 cc. This value, based upon a normal dog hemoglobin of 14 g/100 cc would represent only 0.63% of the total circulating hemoglobin.

Discussion. Ginzel(7) has stressed the importance of evaluating the therapeutic activity of mephenesin-like compounds in ratio to their hemolytic effect which limits their usefulness by the intravenous route. By this comparison GG, which has only slightly less anti-convulsant action than mephenesin, shows a decided advantage over mephenesin by the parenteral route. AHR-85, though somewhat less active immediately than mephenesin, shows marked advantage when the longer duration of action is considered(15).

These data *in vitro* and *in vivo* suggest that both GG and AHR-85 can be given intravenously in large doses (equivalent to 7 g of mephenesin I.V. in a 70 kg man) with little danger of an appreciable hemolytic reaction. The maximal value observed for the serum bilirubin was 0.4 mg%. This is only slightly above the normal human range (0.1 to 0.25 mg%). The maximum observed serum hemochromogen value did not exceed the human renal threshold for hemoglobin (100 mg%)(19) and represents hemolytic liberation of less than 1% of the normal total hemoglobin. The ratio of hemolytic activity *in vitro* between mephenesin and GG which Ginzel *et al.*(8) reported was based on total (100%) hemolysis and a 24 hour test. His finding of a 4:1 ratio of hemolytic activity favorable to GG is comparable to our *in vitro* value determined by HC_{50} 's at 1 hour and 25°C. Berger *et al.*(14) found this ratio to be 3.68:1 when comparisons of 75% hemolytic concentrations were made after 100 minutes. No comparable basis exists for comparing the *in vivo* results reported herein with other hemolytic data on mephenesin *in vivo* (5,6).

Summary. Mephenesin, guaiacol glyceryl

ether (GG) and methocarbamol (AHR-85) have been compared as to their relative hemolytic activity *in vitro* and *in vivo*. Evidence is presented that GG and AHR-85 may be administered slowly as saturated aqueous solutions in large doses with little danger of an appreciable degree of hemolysis as indicated by the serum bilirubin and hemochromogen tests.

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Metabolism of Arterial Tissue. Oxidative Capacity of Intact Arterial Tissue.* (23241)

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In contrast to the extensive literature dealing with chemical changes in circulating blood in atherosclerosis, the role played by the metabolism of arterial tissue in atherogenesis has received little attention. It is known that while arterial tissue is susceptible to either spontaneous or experimental atherosclerosis in certain species, some arteries are more affected by it than others, and moreover in the same artery, such as the aorta, there are sites of predilection for its development. Investigation of the metabolism of arterial tissue in various species and in various segments of the aorta may be helpful in understanding at least partly, the reason for the site of predilection of this pathologic process. The study of metabolic patterns of the 3 aortic segments (ascending and arch, referred to hereafter as arch, descending thoracic, and abdominal) in man, rabbit and dog was therefore undertaken.

Our investigation deals with the oxidative capacity of normal aortic tissue, namely succinic oxidase and cytochrome oxidase systems, in tissue slices, as determined by oxidative response to succinate and p-phenylenediamine. Study of these 2 enzymatic systems appeared of particular interest since it was shown in various tissues that succinic dehydrogenase, cytochrome oxidase(1) and cytochrome c (2), among others(3), are affected by the level of thyroid function, which in turn plays a role in rendering certain species susceptible to atherosclerosis(4).

Material and methods. The tissues used were aorta of man, rabbit and dog. Liver and inferior vena cava (infra-renal segment) were also used for comparison. Animal specimens were secured from male chinchilla rabbits and from male and female mongrel dogs. Animals were sacrificed either by exsanguination or air injection. Human specimens were se-

cured from autopsies within a few to 18 hours after death, during which time they were kept in refrigerators. Preliminary experiments with animal tissues, simulating conditions before autopsy, were performed to ascertain the validity of this study after this lapse of time. Tissues kept at room temperature for 3 to 4 hours, then kept at $+4^{\circ}\text{C}$ for about 16 hours, and kept again at room temperature for 2 to 3 hours, showed the same oxidative capacity as that determined in tissues shortly after removal from the animal, except for endogenous oxygen uptake which became negligible or very small. The used segments of human aortic tissue were free of atherosclerosis. After removal from the host, the tissues were immediately placed in ice-cooled containers with tight-fitting tops to avoid evaporation. The blood vessels were carefully freed of adherent fat and connective tissue, rinsed quickly in ice-cold saline solution and blotted with filter paper. The study of aortic tissue was performed on its 3 segments, *i.e.* arch, descending thoracic and abdominal. Slices of liver and aorta were prepared free-hand by razor blade, while the tissue was placed between 2 frosted glass plates, the bottom one lying on crushed ice. Thin blood vessels, such as rabbit's aorta and inferior vena cava, were opened along the longitudinal axis and cut transversally to provide strips of tissue. The slices or strips were collected in ice-cooled chambers, blotted, weighed and transferred to ice-cooled manometer flasks containing Krebs-Ringer phosphate solution, pH 7.4(5) of final phosphate concentration 0.023 M, in the main chamber and the substrate in the side arm. The thickness of slices was about 0.3-0.5 mm for liver and about 0.5-0.7 mm for aorta. Preliminary experiments with aortic tissue slices of various thicknesses have shown that up to 1 mm the thickness of the slice was not the limiting factor in its oxygen uptake. Oxygen consumption was determined by the direct method of Warburg(6), at

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TABLE I. Oxidative Capacity of Aorta, Inferior Vena Cava and Liver of Man, Rabbit and Dog. Substrate: succinate.

Species		Aorta			Vena cava	Liver
		Arch	Thoracic	Abdominal		
Man	QO ₂ †	.61 (.23-1.65)*	.73 (.27 1.62)	.57 (.17-1.02)	1.62 (.85-2.32)	15.00 (4.37-25.63)
	Determinations	18	20	19	12	15
Rabbit	QO ₂	2.19 (1.81-2.47)	2.21 (1.54-2.71)	2.10 (1.34-2.64)		19.28 (13.23-35.18)
	Determinations	11	20	15		15
Dog	QO ₂	2.84 (2.00-3.44)	2.59 (1.92-3.58)	1.87 (1.06-3.13)	.56 (.39- .84)	36.76 (20.84-44.91)
	Determinations	20	19	15	10	16

* Figures in parentheses represent range of variation.

† QO₂ = Oxygen consumption in mm³/mg initial dry wt/hr, corrected for oxygen uptake of tissue in absence of added substrate.

37.5°C, with oxygen as gas phase. Amounts of fresh tissue/flask were 100-150 mg for rabbit and dog aorta, 150-200 mg for human aorta, 125-150 mg for inferior vena cava, 30-40 mg for rabbit and human liver, and 20-30 mg for dog liver. The aorta of man and dog provided sufficient material for duplicate determinations whereas that of the rabbit, particularly arch and abdominal segments, did not provide enough material, making it necessary to pool tissues of 2 to 3 rabbits. Initial dry weights were determined for each experiment, by drying tissues to constant weight at 106°-108°C. Substrates used were sodium succinate (pH 7.4) of final concentration 0.05 M for determination of the succinic oxidase system and p-phenylenediamine of final concentration 0.03 M, dissolved in phosphate buffer (pH 7.4) under nitrogen, for the cytochrome oxidase system. Their concentration appeared to be optimal since rate of oxygen consumption remained constant when the concentration was doubled, and it increased proportionately with increasing amounts of tissue. Both these substrates are known to penetrate the tissues. In the presence of succinate, oxygen uptake was constant during 30-40 minutes for liver and 40-60 minutes for artery and vein. In the presence of p-phenylenediamine it was constant during 10-20 minutes for liver and 40-60 minutes for artery and vein. The oxygen consumption/hour was computed from the period of linearity. It was related to initial dry weight and was expressed as QO₂, *i.e.* oxygen

consumption in cmm/mg initial dry weight/hour, corrected for oxygen uptake of tissue in absence of added substrate.

Results. Tables I and II summarize the results obtained with the 3 aortic segments and liver of man, rabbit and dog, and inferior vena cava of man and dog. All ages were included in these groups. They varied from one day to 73 years for man, 5 months to 2½ years for the rabbit and 5 months to 17 years for the dog.

These Tables reveal that in man and rabbit the 3 aortic segments showed no appreciable difference in oxidative response to either succinate or p-phenylenediamine. In contrast, in the dog, while the 2 first aortic segments exhibited no appreciable difference in their oxidative response to both substrates, the abdominal segment showed a markedly lower response to succinate and a moderately lower to p-phenylenediamine. It is noteworthy that in man the oxidative response of the inferior vena cava to both substrates was about 3 times higher than that of the aorta, whereas in the dog it was about 3-5 times lower with succinate and 2 times lower with p-phenylenediamine. The oxidative response of arterial and venous tissues to both succinate and p-phenylenediamine appears to be quite low in the 3 species studied, when compared to that of the liver. The oxidative response of aortic and venous tissues to succinate was approximately 1/24th and 1/9th that of liver in man, 1/13th, 1/20th and 1/65th for the first 2 aortic segments, the ab-

TABLE II. Oxidative Capacity of Aorta, Inferior Vena Cava and Liver of Man, Rabbit and Dog. Substrate: p-phenylenediamine.

Species		Aorta			Vena cava	Liver
		Arch	Thoracic	Abdominal		
Man	QO ₂ †	.67 (.33-1.31)*	.74 (.28-1.29)	.71 (.43-1.26)	2.20 (1.24-3.01)	11.26 (4.93-17.00)
	Determinations	17	20	18	12	14
Rabbit	QO ₂	2.34 (1.63-3.13)	2.42 (1.81-3.10)	2.35 (1.80-3.04)		13.07 (9.78-20.80)
	Determinations	12	18	12		15
Dog	QO ₂	2.32 (1.49-2.95)	2.53 (1.50-4.58)	2.09 (1.27-3.42)	1.38 (1.12-1.57)	27.56 (15.48-33.45)
	Determinations	20	18	15	10	16

* Figures in parentheses represent range of variation.

† QO₂ = Oxygen consumption in mm³/mg initial dry wt/hr, corrected for oxygen uptake of tissue in absence of added substrate.

dominal aorta and inferior vena cava respectively in the dog, and 1/9th for the 3 aortic segments of the rabbit. Using p-phenylenediamine as substrate, the oxidative response of aortic and venous tissues was approximately 1/15th and 1/5th that of liver in man, 1/13th and 1/20th respectively in the dog and 1/5th for the aortic tissue in the rabbit.

Influence of age. Values of oxidative responses to succinate and p-phenylenediamine summarized in Tables I and II represent averages of results obtained in groups including all ages. When correlated with age, it was found that in the rabbit (5 months to 2½ years) and in the dog (5 months to 17 years) there was no appreciable change in oxidative responses to both substrates in either aorta, liver or vena cava. In contrast, in man, the oxidative capacity diminishes with age in aortic tissue while showing no appreciable change in vena cava and liver. Tables III

and IV summarize the influence of age on oxidative capacity of the 3 aortic segments, inferior vena cava and liver, in man. These Tables reveal that average values of oxidative responses of the aortic tissue to succinate and p-phenylenediamine are markedly lower in the group 21 to 73 years old, as compared to those of the group one day to 17 years old. Table III also reveals that the oxidative response to succinate is lower in the abdominal aortic segment in the younger group, and that this difference between thoracic and abdominal aorta seems to level off in the older group. The oxidative response of aortic tissue to succinate in the older group is about 50% and 30% lower in the thoracic (arch and descending) and abdominal segments respectively as compared to that found in the younger group. Using p-phenylenediamine as substrate, oxidative response of the 3 aortic segments in the older group is about

TABLE III. Comparison of Oxidative Capacity of Aorta, Inferior Vena Cava and Liver of Man One Day to 17 Years and 21 to 73 Years Old. Substrate: succinate.

Age		Aorta			Vena cava	Liver
		Arch	Thoracic	Abdominal		
1 day - 17 yr	QO ₂ †	1.00 (.49-1.65)*	1.17 (.57-1.63)	.72 (.38- .93)	1.54 (.90-1.96)	16.29 (4.37-25.63)
	Determinations	6	8	8	5	6
21-73 yr	QO ₂	.44 (.23- .75)	.50 (.27- .90)	.53 (.17-1.02)	1.66 (.85-2.32)	14.13 (8.58-24.50)
	Determinations	12	12	11	7	9

* Figures in parentheses represent range of variation.

† QO₂ = Oxygen consumption in mm³/mg initial dry wt/hr, corrected for oxygen uptake of tissue in absence of added substrate.

TABLE IV. Comparison of Oxidative Capacity of Aorta, Inferior Vena Cava and Liver of Man One Day to 17 Years and 21 to 73 Years Old. Substrate: p-phenylenediamine.

Age		—Aorta—			Vena cava	Liver
		Arch	Thoracic	Abdominal		
1 day- 17 yr	QO ₂ †	.98 (.79-1.31)*	1.07 (.79-1.29)	1.08 (.96-1.26)	2.20 (1.40-3.01)	12.20 (5.01-17.00)
	Determinations	6	8	6	6	7
21-73 yr	QO ₂	.55 (.33-.79)	.45 (.28-.63)	.59 (.43-.69)	2.20 (1.24-2.61)	10.45 (4.93-15.88)
	Determinations	11	12	12	6	7

* Figures in parentheses represent range of variation.

† QO₂ = Oxygen consumption in mm³/mg initial dry wt/hr, corrected for oxygen uptake of tissue in absence of added substrate.

50% lower.

Discussion. Oxidative response of arterial tissue slices to succinate and p-phenylenediamine in optimum concentration, may be considered to be a fair index of oxidative capacity of the succinic oxidase (succinic dehydrogenase + cytochrome c + cytochrome oxidase) and cytochrome oxidase (cytochrome c + cytochrome oxidase) systems and affords a quantitative measure of their oxidative capacity, with cofactors available at the concentration existing in the tissues. This method has proven of value in the study of a number of normal and neoplastic tissues(7,8).

Oxidative utilization of succinate indicates the existence of succinic dehydrogenase in the aorta of man, rabbit and dog and vena cava of man and dog. The vena cava of the rabbit was not studied. Our results are in agreement with previous findings of the existence of this enzyme in the thoracic aorta of rat(9) and man(10). The oxidative response to p-phenylenediamine indicates the presence of cytochrome oxidase in the above mentioned vessels.

Our findings reveal that aortic and venous tissues possess a relatively low oxidative capacity for both succinic and cytochrome oxidase systems. Our data further indicate that a difference exists in oxidative capacity of aortic tissue in regard to the above 2 systems, in the 3 species studied, human aorta exhibiting the lowest oxidative response to both substrates. The dog's thoracic aorta exhibits the highest response to succinate, while its abdominal segment and the 3 aortic segments of the rabbit exhibit values of equal magnitude,

between those of man and thoracic aorta of the dog. The oxidative response to p-phenylenediamine is of equal magnitude in the aorta of rabbit and dog.

Paralleling these findings, the liver shows the lowest oxidative capacity in man, highest in the dog and intermediate in the rabbit. In contrast, the oxidative response of human vena cava to both substrates is higher than that of the dog.

A similar species difference exists for adenosine triphosphatase, which is higher in the aortic tissue of the dog as compared to that of the rabbit(11). Oxygen uptake of the aorta is also different in several species, in a medium containing simultaneously lactate, glutamate and fumarate(12).

Since the aorta is composed of several layers of different tissues, relationship between enzymatic makeup of aortic tissue and its morphologic components may be raised both with regard to its segments and species. Histologic differences between thoracic and abdominal aorta are known to exist and consist chiefly in greater thickness of the media and more abundant elastic tissue in the thoracic segment. While in the dog, it may be tempting to ascribe differences in oxidative responses of thoracic versus abdominal segment to the above morphologic differences, this does not hold true, however, in rabbit and man. Likewise, the observed differences in oxidative responses of aortic tissue in the 3 species studied do not seem to reflect the known difference in morphologic components. Factors responsible for the above species difference remain to be elucidated.

The lower oxidative capacity of the succinic oxidase system in the abdominal aorta of the dog, as compared to the thoracic, may prove of interest when correlated to its susceptibility to atherosclerosis. Indeed, in the dog on an atherogenic regimen, the abdominal segment is the most susceptible to atherosclerosis, whereas the thoracic aorta is refractory[†](13, 14). The decrease in oxidative capacity of human aorta with age may prove equally interesting when correlated to the known greater susceptibility of man to atherosclerosis with advancing age. It is likely that the observed differences between the 3 species studied may be part of a broader difference in metabolic patterns of their arterial tissue. Work now in progress seems to support this view.

Summary. The oxidative capacity of succinic oxidase and cytochrome oxidase systems in 3 aortic segments (ascending and arch, descending thoracic, abdominal), inferior vena cava and liver slices of man, rabbit and dog was studied. A species difference was found to exist: a) man exhibited the lowest oxidative values for both systems; b) the dog, the highest values for the succinic oxidase system in the thoracic aorta; c) the rabbit, intermediate values for the succinic oxidase system in all 3 aortic segments; d) oxidative values for the cytochrome oxidase system were of approximately equal magnitude in the aortic tissue of dog and rabbit. A significant decrease in oxidative capacity of aortic tissue

was associated with aging in man, while the vena cava and liver remained unaffected. No appreciable change with age was found in rabbit and dog.

The significance of the above findings in relation to atherogenesis was discussed.

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Metabolism of the Protein Moiety of Rabbit Serum Lipoproteins. (23242)

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Virtually all of the serum lipids exist in combination with proteins(1). On the basis of electrophoretic mobility(2), solubility(3, 4), and density(5) these lipoproteins may be

divided into two large groups, the alpha or high density lipoproteins and the beta or low density lipoproteins. Each of these groups may be further subdivided on the basis of density differences(6). In addition to these physical differences, the various lipoproteins differ in their lipid composition(7). Recent

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studies have also demonstrated differences in the N-terminal groups of the proteins in the major lipoprotein fractions(8). That these various lipoproteins may also have different functions has been suggested by changes in their concentrations following fat feeding(9) or heparin induced clearing(10). Studies on the relative turnover rates and metabolic interrelationships of the protein moieties of the major lipoprotein fractions should contribute toward further clarification of the function of the various lipoproteins.

Methods. Labeled materials. 1-C¹⁴-alanine was obtained from Tracerlab, Inc. BaC¹⁴O₃ was obtained from Oak Ridge National Laboratories. In certain of the experiments high specific activity C¹⁴-labeled proteins were obtained by growing *Chlorella pyrenoidosa*[†] in an artificial nutrient medium with NaHC¹⁴O₃ as the sole carbon source(11). The organisms were harvested, extracted with hot ethanol, and dried. 1-C¹⁴-alanine and labeled lipoprotein fractions were injected into rabbits weighing 3-4 kg. Samples of the protein obtained from C¹⁴-labeled *Chlorella* were administered by stomach tube in water suspension. **Plasma Fractionation.** Blood samples (6-12 ml) were drawn into heparinized tubes or syringes and immediately centrifuged. The lipoproteins were fractionated according to the procedure of Havel, Eder and Bragdon (7). The plasma was brought to density 1.063 (D 1.063) by addition of the calculated volume of concentrated salt solution (NaCl + NaBr, D 1.346) and centrifuged at 105,000 x g for 16-18 hours at 12-15°C in a Spinco Model L preparative ultracentrifuge. The lipoproteins concentrated in the upper 2 cm³ of the tube (*D* < 1.063 fraction) were separated from the infranatant by the use of a tube slicing device. The infranatant was brought to D 1.21 by the addition of solid KBr and appropriate adjustment of the final volume. After 20 hours centrifugation at 114,400 x g the top layer (*D* 1.063 - 1.21 fraction) was separated by means of the tube slicer. The infranatant was designated the *residual protein fraction* (*D* > 1.21). The

TABLE I. Specific Activity of Serum Fractions after Intravenous 1-C¹⁴-alanine.

Time (hr)	D < 1.063 Fraction	D 1.063-1.21 Fraction	D > 1.21 Fraction
	Counts/min./mM BaCO ₃ *		
2	3300		545
6	3780	680	771
24	840	1090	963
72	610	562	548
120	250	360	417
168	193	140	527
264			242

* Spec. activity of ninhydrin-labile C¹⁴O₂ counted in the form of BaC¹⁴O₃ (see *Methods*).

lipoprotein fractions used for injection into recipient rabbits were dialyzed for 16 hours at 4°C against large volumes of 0.9% NaCl in a rocking continuous-flow dialysis apparatus. **Measurements of specific activity.** In the experiments in which 1-C¹⁴-alanine was administered, the lipoprotein fractions, after dialysis to remove any free amino acids, were hydrolyzed for 16 hours at 110°C in 6 N HCl. CO₂ was liberated from the amino acids by reaction with ninhydrin by the method of Van Slyke, *et al.*(12). The CO₂ was precipitated as BaCO₃ and plated for radioassay. In the experiments in which labeled algal protein was the source of C¹⁴, the lipoproteins were precipitated with 10% trichloroacetic acid, washed with alcohol-ether and then with ether. The washed proteins were hydrolyzed with HCl and the mixture of amino acids was evaporated to dryness repeatedly to remove HCl. The amino acid hydrochlorides were plated directly for radioassay. Radioassays were carried out using the Robinson gas flow proportional counter(13). Results were corrected for self-absorption to a weight of 5 mg using a curve derived for BaCO₃. As shown by Karnovsky *et al.*, the self-absorption curves for organic compounds in the Robinson counter differ very little from that for BaCO₃(14).

Results. Injection of 1-C¹⁴-alanine. 100 µc (8.9 mg) of 1-C¹⁴-alanine was injected intravenously. The specific radioactivities of the various fractions as a function of time are shown in Table I. The D < 1.063 fraction showed an initial specific activity considerably higher than that in the other two fractions and reached maximal activity earlier.

[†] We are indebted to Dr. Rodney A. Olson for the *Chlorella* strain used.

The D 1.063 - 1.21 lipoproteins and the residual proteins attained lower levels of activity and maximum activity was reached between 6 and 24 hours. Half-time values estimated on the basis of the points beyond 24 hours were: D<1.063 fraction, 60-70 hours; D 1.063-1.21 fraction, 50-60; D>1.21 fraction, 120-140 hours. During the early period the specific activity of the D<1.063 fraction decreased rapidly with half maximal activity reached at 9 hours. Because the repeated bleedings necessary for these studies disturbed the steady state, the true disappearance rates are probably somewhat lower. It is clear, however, that the circulating lipoproteins turn over more rapidly than the remaining serum proteins considered as a group. In a subsequent experiment the D<1.063 fraction was subfractionated into a D<1.006 and a D 1.006-1.063 fraction. The specific activities of the protein in the two fractions were identical at the two points studied.

Fate of C¹⁴-labeled lipoprotein fractions. In the first series of experiments the labeled lipoproteins were prepared from a donor rabbit given 480 μ c (68.1 mg) of 1-C¹⁴-alanine intravenously. Six hours later the animal was exsanguinated and the D<1.063, D 1.063-1.21, and D>1.21 fractions were isolated and prepared for intravenous injection into recipient rabbits, as described previously. Enough of each fraction was injected to double the circulating level of this fraction in the recipient animal. Blood samples were taken at frequent intervals up to 96 hours. In each animal the administered protein disappeared rapidly from the circulation. By 30 hours virtually all of the injected D<1.063 protein had disappeared from the circulation of the recipient animal. The D 1.063-1.21 and D>1.21 fractions disappeared at a slower rate and remained at low levels in the circulation for over 96 hours. In each recipient animal protein fractions other than the one injected showed some activity, but the relative activities of these other fractions did not vary consistently with time, as would have been the case had interconversion of one protein species to another occurred. Experiments were therefore undertaken to test for the completeness of fractionation under the condi-

TABLE II. Relative Specific Radioactivities of Lipoprotein Fractions after Mixing Labeled D>1.21 Fraction with Whole Serum.

	D <1.063	D 1.063-1.21	D >1.21
Procedure A	2.4	7.2	100
B	.9	2.1	"
C	.6	1.7	"

tions of ultracentrifugation used. A labeled D>1.21 fraction, which was presumably free of lipoproteins, was mixed *in vitro* with normal whole rabbit plasma and the mixture was refractionated by ultracentrifugal flotation. The D<1.063 and the D 1.063 - D 1.21 fractions, which should have been free of radioactivity, contained protein of specific activity respectively 22% and 28% of that in the D>1.21 fraction. In the rabbit only 26 mg per 100 ml of total serum protein is found in the D<1.063 fraction(6). Consequently, the presence of less than 0.2% of the protein of the D>1.21 fraction remaining in the D<1.063 fraction would give the observed specific activity.

To see if further centrifugation would eliminate this apparent contamination, rabbit serum labeled by feeding C¹⁴-Chlorella was centrifuged twice at D>1.21 and the infranant was then mixed with whole unlabeled rabbit serum. The mixture was fractionated as in the routine preparative procedure. From the D<1.063 and the D 1.063 1.21 fractions aliquots were removed for determination of protein specific activities (Table II, Procedure A). The remainder of each fraction was divided into two aliquots. One aliquot of each fraction was again centrifuged overnight (Procedure B). The other was mixed with unlabeled D>1.21 fraction and centrifuged overnight (Procedure C). As shown in Table II, the centrifugations, both with and without addition of the unlabeled D>1.21 fraction, markedly reduced the contamination of both lipoprotein fractions with D>1.21 proteins but did not completely remove it. Apparently the sedimentation of proteins of density greater than 1.21 is not complete under these conditions. When labeled D<1.063 or labeled D 1.063 1.21 lipoproteins were added to whole serum and fractionated as above, cross contamination between the frac-

TABLE III. Fate of C¹⁴-Labeled Serum Fractions in Recipient Rabbits.

Labeled fraction	Time after inj. (hr)	Specific radioactivity of protein (cpm/mg)		
		D <1.063 fraction	D 1.063-1.21 fraction	D >1.21 fraction
D <1.063	<i>In vitro</i> *	4,592 (100)†	233 (5.1)	.0 (0)
	Inj. 1	280 (")	10 (3.7)	.0 (0.1)
	4	154 (")	16 (10.0)	.1 (")
	24	36 (")	1 (3.9)	.6 (2.5)
D 1.063-1.21	<i>In vitro</i>	23 (7.3)	318 (100)	.6 (0.2)
	Inj. 4	7 (2.9)	331 (")	1.0 (0.3)
	24	1 (1.0)	155 (")	1.2 (0.8)
D >1.21	<i>In vitro</i>	3 (1.1)	36 (14)	262 (100)
	Inj. 1	19 (5.1)	54 (28)	191 (")
	24	7 (8.0)	14 (15)	89 (")

* 1-C¹⁴-alanine labeled D <1.063 lipoprotein from another experiment was used in this fractionation control and centrifuged only once.

† Figures in parentheses are relative specific activities with that of the fraction used for inj. arbitrarily assigned a value of 100.

tions also occurred, but to a lesser extent (Table III, "*in vitro*"). In view of these findings, all fractions used for injection in the subsequent experiments were centrifuged twice.

In Table III is summarized an experiment in which protein-labeled fractions were injected into recipient animals. Control data are shown giving results on the distribution of radioactivity in each fraction after *in vitro* mixing of an aliquot of the injected fraction with whole serum. The injected fractions were prepared from the sera of animals fed C¹⁴ algal proteins. Sera from the recipient animals were obtained at the indicated time intervals after injection of the labeled protein, and the specific activity of the protein in each of the three fractions was determined. The rate of disappearance of the injected protein was rapid. In the animal receiving the D <1.063 fraction the activity in the other two fractions was not significantly different from that found after *in vitro* mixing. This was true also of the animal receiving the D 1.063 - 1.21 fraction. In the animal receiving the D >1.21 fraction, the activities in the other two fractions were higher than found with *in vitro* mixing.

Discussion. The findings that the protein moiety of the lipoprotein fractions turns over at a rate faster than that of the other plasma proteins and that the protein of the low density lipoproteins turns over at a rate

greater than that of the high density lipoproteins is in agreement with the observations of others. Volwiler, *et al.*(15) labeled serum lipoproteins in man by feeding S³⁵ cystine and observed a $t_{1/2}$ for the beta lipoproteins ranging from 3 to 7 days, with a median value of 6.1 days. Using proteins labeled with I¹³¹ Gitlin and Cornwell(16) found a $t_{1/2}$ for the S_f 3-8 class of lipoproteins of about 3 days and for the alpha lipoproteins of over 4 days.

Most studies on the metabolic relationships between lipoproteins have been indirect. In the ultracentrifugal studies of Graham *et al.* (10), falling concentrations of low density lipoproteins (S_f 20-100) during heparin induced clearing were correlated with simultaneous increases in higher density lipoproteins (S_f 12-20). Recently, direct evidence for transfer of proteins from the S_f >8 class to the S_f 3-8 class has been presented by Gitlin and Cornwell(16), using I¹³¹ labeled lipoproteins. They found no evidence for conversion of these classes of lipoproteins to alpha lipoproteins. The present studies are not exactly comparable since interconversion within the D <1.063 fraction was not studied. This present study shows no evidence for significant conversion of D <1.063 lipoproteins to D 1.063 - 1.21 lipoproteins or vice versa. These results are consonant with previous studies showing that the proteins in these fractions have distinctly different chemical structure(8) and physical properties

(1,17). Studies of conversion of $D > 1.21$ proteins to lipoproteins are inconclusive because of the necessity of making almost complete separation between these proteins and the lipoproteins. Furthermore, in the *in vivo* studies where these proteins are injected, relatively large amounts of labeled amino acids are present and these may well be reutilized in the synthesis of lipoproteins.

Summary. Alanine-1- C^{14} was administered to rabbits and its incorporation into the lipoproteins of $D < 1.063$ and $D 1.063 - 1.21$ fractions and into the remaining serum proteins was measured. The $D < 1.063$ fraction reached a higher specific activity than did the $D 1.063 - 1.21$ lipoprotein fraction or the $D > 1.21$ fraction. Both lipoprotein fractions turned over at a faster rate than did the remaining serum proteins. C^{14} -labeled lipoprotein fractions were injected into rabbits and there was no significant interconversion between the two major density classes of lipoproteins studied. These data suggest that the $D < 1.063$ and $D 1.063 - 1.21$ classes of lipoproteins are metabolically as well as chemically distinct.

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Anti-fibrillatory Activity of 17-(2-Piperidylmethyl)- 3β , 17 β -androstane diol. (23243)

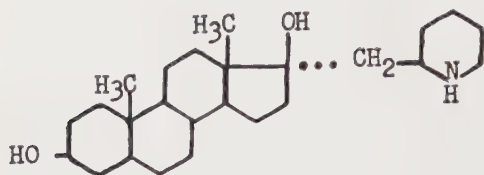
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A number of basic steroids have been found to possess cardiac activity. Krayner, Uhle and Ourisson(1) showed that 20-(5'-methyl-2'-piperidyl)-5-pregnene-3, 20 diol had anti-accelerator action on the heart-lung preparation of the dog. Margolin, Lu, Yelnosky and Makovsky(2) demonstrated both anti-accelerator and anti-fibrillatory activity for 16-

cyclohexylamino-allopregnandiol in the dog, while Robson and Trounce(3) found that 16-dimethylaminomethyl - epi-dehydroisoandrosterone antagonized ventricular arrhythmias in the cat. The present report describes the anti-fibrillatory activity of 17-(2-piperidylmethyl)- 3β , 17 β -androstane diol (Ro 2-7302). This compound has the following

structure:



Methods. 1. *Chemical:* The 17-(2-piperidylmethyl)-3 β , 17 β -androstane diol used in this investigation was prepared by condensation of dehydroisoandrosterone with 2-picolyl lithium, followed by catalytic hydrogenation of the resulting 17-(2-pyridylmethyl)-5-androstene-3 β , 17 β -diol. This latter compound has recently been described by Heer and Hoffmann(4), who obtained it by a slightly different method. Our purified 17-(2-pyridylmethyl)-5-androstene-3 β , 17 β -diol melts at 179-183° (corr.), while Heer and Hoffmann (4) report a melting point range of 178-185°. Catalytic hydrogenation of the 17-(2-pyridylmethyl)-5-androstene-3 β , 17 β -diol was carried out as follows: To 17.2 g of 17-(2-pyridylmethyl)-5-androstene-3 β , 17 β -diol in 300 ml of methanol was added 8 ml of 6 N hydrochloric acid and the mixture hydrogenated in the presence of 1.6 g of platinum oxide catalyst at 100 lb/sq. in. and 50°C. After the hydrogen uptake had ceased, the catalyst was separated by filtration and the filtrate evaporated to dryness *in vacuo*. The residue so obtained was slurried with water, made alkaline and extracted with chloroform. Evaporation of the dried chloroform extract gave a residue, which on crystallization from ethyl acetate or from acetonitrile yielded 17-(2-piperidylmethyl)-3 β , 17 β -androstane diol, melting at 211-213°C. (corr.). Calcd. for C₂₅H₄₃O₂N: C, 77.07; H, 11.13. Found: C, 76.95; H, 10.84. Although this compound was not reported by Heer and Hoffmann(4), they do describe its 3-monoacetyl derivative. The sulfate (decomp. >320°), the d-camphor-10-sulfonate (decomp. 236-239°), the maleate (m.p. 126-160°) and the d-tartrate (decomp. 242-249°) all crystallize with one mole of water, whereas the hydrochloride (decomp. >320°) and the lactate (m.p. 120-123°) are hemihydrates. Only the lactate is soluble in water; this form (Ro 2-7302/4)

was therefore used in the following experiments.

2. *Pharmacological:* As the technics have already been described in detail(5,6), only brief mention of them will be made here. *The isolated rabbit auricle test* (Dawes, 7) is based on measurement of the maximum rate of electrical stimulation which the auricles can follow; this rate is lowered by quinidine. Drug effects are indicated in terms of the greatest change in maximum frequency occurring during one hour's exposure to the drug. Only one drug test was conducted on each heart. *Auricular fibrillation in the dog* was induced by placing a cotton pledget soaked in 5% acetylcholine (ACh) on the area of the sinus node (Scherf and Chick, 8). After several control fibrillations (average duration 18 minutes), the drug being tested was injected, and its effect on duration of fibrillation noted. In addition to intravenous administration, absorption of drugs from the gastrointestinal tract was studied by injection into the duodenum. *Aconitine-induced arrhythmias* were studied in the dog by placing a pledget soaked in 5% aconitine on the sinus node area for 30 seconds (Prinzmetal *et al.*, 9). This produced a mixture of auricular flutter and auricular fibrillation with occasional ventricular ectopic beats. As the transition to and from these arrhythmias was gradual, it was difficult to determine their duration; however, the accompanying increase in heart rate was easily measured. The drugs being tested were injected 1 min. before application of aconitine; 3 control animals were injected with saline in the same manner. The figures in Table I show percentage increase in heart rate following aconitine. *Ventricular fibrillation* in the dog was induced by sensitizing the myocardium with chloroform, followed by epinephrine (Epi) 0.01-0.02 mg/kg I.V. (Melville, 10). This procedure resulted in ventricular fibrillation and death in 5 out of 8 control animals. Eight other dogs were injected with quinidine and 9 with Ro 2-7302/4, each animal receiving only a single dose of drug. *Electrocardiograms* were recorded in separate experiments. Three dogs received quinidine in successive doses of 5, 10, and 20

TABLE I. Anti-Fibrillatory Activity.

No. of animals per dose	Test	Dose of drug	Quinidine sulfate	Ro 2-7302/4
4	Isolated rabbit auricles	mg/l	% change in max frequency	
		.5	- 6 \pm 6	-12 \pm 4
		1	-22 \pm 9	-19 \pm 6
		5	-39 \pm 4	-42 \pm 11
3	ACh-induced auricular fibrillation in dog	mg/kg	% change in fibrillation duration	
	Intrav. admin.	.125		-19 \pm 35
		.25		-88 \pm 24
		.50		-93 \pm 15
		2	0 \pm 39	
		4	- 71 \pm 29	
		8	- 97 \pm 5	
	Intra-duodenal admin.	20	-100 \pm 0	-96 \pm 4
3	Aconitine-induced tachycardia in dog	mg/kg I.V.	% change in heart rate	
		10	+70 \pm 70	+ 25 \pm 40
			Saline controls,	+135 \pm 75
2-4	Epi-induced ventricular fibrillation in dog	mg/kg I.V.	No. dogs fibrillated/No. dogs tested	
		1	1/2	
		2.5	0/2	0/2
		5	0/2	2/3
		10	0/2	3/4
		Total	1/8	5/9
		Saline controls		5/8

mg/kg I.V. with an interval of 1 to 2 hours between injections. Three other dogs received Ro 2-7302/4 by the same schedule. All experiments on dogs were performed under pentobarbital anesthesia; only one anti-fibrillatory drug was administered to each animal.

Results. The effects of quinidine sulfate and Ro 2-7302/4 on experimentally induced arrhythmias are compared in Table I. The drugs were equally active in depressing the maximum rate at which the isolated rabbit auricles can be driven. Ro 2-7302/4 was approximately 16 x as active as quinidine in antagonizing ACh-induced auricular fibrillation in the dog, since its action at 0.5 mg/kg I.V. was about the same as that of quinidine at 8 mg/kg. Both drugs antagonized ACh-induced auricular fibrillation when injected into the duodenum at a dose of 20 mg/kg. Dogs treated with Ro 2-7302/4 at 10 mg/kg I.V. showed less increase in heart rate following aconitine than did those treated with quinidine at the same dose. On the other hand, quinidine was more active than Ro 2-7302/4 in preventing Epi-induced ventricu-

lar fibrillation in the dog.

Effects of these drugs on the ECG of the dog are illustrated in Fig. 1. Blood pressure, heart rate and ECG data are given in Table II. Both quinidine and Ro 2-7302/4 at doses of 10 and 20 mg/kg I.V. produced bradycardia, hypotension, increase of P-Q, QRS, and Q-T durations, and decrease in amplitude of R wave. Quinidine increased amplitude of the S wave at all doses; Ro 2-7302/4 increased amplitude of this wave at 5 mg/kg I.V., had little effect at 10 mg/kg, and decreased amplitude at 20 mg/kg. Amplitude of the T wave was progressively increased with increasing doses of quinidine, whereas with increasing doses of Ro 2-7302/4 it was first decreased and then reversed.

Other cardiovascular effects: Neither quinidine nor Ro 2-7302/4 at doses of 1 and 4 mg/kg I.V. had any effect on the blood pressure changes induced by ACh, Epi, serotonin, or occlusion of the carotid artery.*

* We wish to thank Miss Tina Ferruggia for making these measurements.

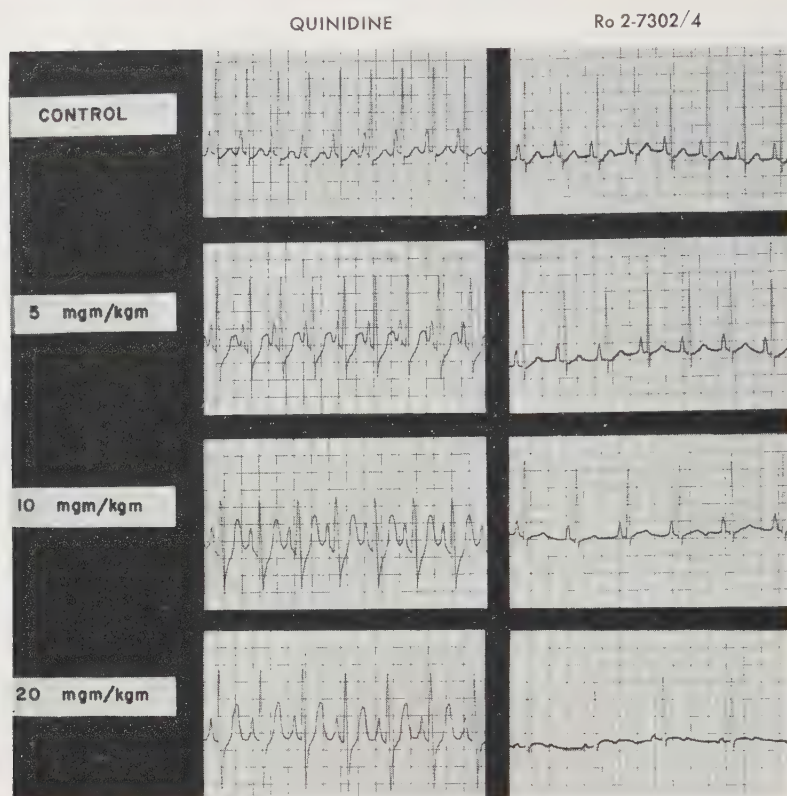


FIG. 1. Effects of drugs on electrocardiogram (Lead II) of dogs under pentobarbital anesthesia. Left: Dog. No. 589. Quinidine, 5, 10, and 20 mg/kg I.V. Right: Dog No. 643. Ro 2-7302/4, same dose levels.

Discussion. Ro 2-7302/4 showed activity equal or superior to that of quinidine on the isolated rabbit auricle, on ACh-induced auricular fibrillation and on aconitine-induced tachycardia in the dog. Both drugs produced side effects, as shown by marked blood pressure and ECG changes, at 10 and 20 mg/kg I.V. Whereas quinidine showed antifibrillatory activity at 4 and 8 mg/kg, Ro 2-7302/4

showed this activity at 0.25 and 0.50 mg/kg. This suggests that the safety margin of Ro 2-7302/4 is greater than that of quinidine. This compound merits further study as a potential anti-fibrillatory agent.

Summary. 17-(2-Piperidylmethyl) - 3 β , 17 β -androstane diol lactate (Ro 2-7302/4) has an activity equal to that of quinidine in diminishing maximum rate of the electrically

TABLE II. Effects of Drugs on Blood Pressure, Heart Rate and ECG (Lead II) of Dog. (Each figure is avg of 3 experiments; readings taken 1 min. after drug inj.)

Drug	Dose, mg/kg I.V.	Percentage change, relative to pre-drug value								
		Heart rate	Blood pressure		Duration			Amplitude		
			Systolic	Diastolic	P-Q	QRS	Q-T	R	S	T
Quinidine sulfate	5	0	-8	-19	+11	-2	+7	-17	+46	+41
	10	-9	-34	-41	+32	+27	+11	-32	+84	+118
	20	-24	-46	-66	+58	+108	+37	-42	+46	+164
Ro 2-7302/4	5	-16	-10	-12	+13	+26	+34	-10	+36	-20
	10	-24	-21	-34	+24	+43	+48	-19	+14	-80
	20	-47	-53	-72	+65	+100	+58	-33	-64	-240

driven isolated rabbit auricle. It is approximately 16 times as active as quinidine in antagonizing acetylcholine-induced auricular fibrillation in the dog. It is slightly more active than quinidine in antagonizing aconitine-induced tachycardia in the same species. The safety margin of this drug, as measured by the ratio between minimal anti-fibrillatory doses and those producing marked blood pressure and ECG changes, is greater than that of quinidine.

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Effect of Tolbutamide (Orinase) on Plasma Non-Esterified Fatty Acids. (23244)

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Oral or parenteral administration of sulfonylurea derivatives produces hypoglycemia in normal subjects and in many diabetic patients(1,2). Since functioning pancreatic tissue is required for this action, it has been suggested that tolbutamide (1-butyl-3-p-tolyl-sulfonylurea) in some manner enhances the effect of endogenous insulin(3,4). On the other hand, there is evidence that tolbutamide may limit release of glucose from the liver into the blood stream(5,6) and thus reduce concentration of blood glucose in this manner rather than by promoting utilization. The close association between metabolism of carbohydrate and the level of plasma non-esterified fatty acids (NEFA) provides another way to test this question. Since an increased oxidation of carbohydrate appears to be associated with a fall of NEFA concentration, it should be possible to reveal any effect of tolbutamide on oxidation of carbohydrate by its action on the NEFA fraction of blood lipides.

Method. Tolbutamide was administered to 4 normal, 7 mild adult diabetic, and 3 se-

vere juvenile-type diabetic patients, in a dose of 2 g dissolved in 20 cc saline and given intravenously over a 2-minute period. Venous blood samples, collected in duplicate before injection and at 20, 40, 60, and 120 minutes thereafter, were analyzed for glucose by a modification of the Nelson colorimetric procedure(7) and for NEFA by a method previously described(8). Urine samples for qualitative determinations of sugar and ketones were obtained at hourly intervals.

Results. Concentrations of blood glucose and NEFA observed in the normal and diabetic patients are given in Table I. In agreement with previous observations, tolbutamide produced hypoglycemia in normals and mild diabetics, but failed to lower blood sugar of the 3 diabetics with histories of recurrent ketosis. A decrease in NEFA occurred in every patient who responded with a reduction of blood sugar; no significant decrease was observed in the absence of a hypoglycemic effect. The correlation between changes in concentration of NEFA and blood sugar was highly significant ($p < 0.001$).

TABLE I. Effect of Tolbutamide on Blood Glucose and Non-Esterified Fatty Acids.

TABLE I. Effect of Toluamide on Blood Glucose and NEFA Levels												
Sex	Age	Wt (kg)	Glucose					NEFA				
			Initial value (mg %)	% change				Initial value (mg %)	% change			
				Min.					Min.			
				20	40	60	120		20	40	60	120
<i>(A) Normal</i>												
* ♀	20	86	93	-11	-35	-37	-18	1215	-9	-42	+6	+37
♀	33	75	78	-26	-42	-26	-15	774	-5	-27	+10	+8
♀	22	84	71	-30	-23	-1	-3	1051	-16	-43	-47	+11
† ♂	26	78	91	-31	-29	-3	-4	648	-31	+27	+41	+8
<i>(B) Mild diabetic</i>												
♂	54	104	160	-8	-13	-13	-20	781	+2	-27	-35	-20
♀	50	77	188	-7	-9	-19	-28	833	+4	-15	-25	-29
♀	54	62	182	+4	+2	-4	-14	1360	-1	-17	-29	-18
♀	35	94	157	-10	-15	-17	-29	758	+5	-13	-18	-9
♂	48	74	159	-17	-35	-42	-52	532	-3	-24	-30	-35
† ♂	50	55	179	-14	+6	+10	+27	1063	0	+5	-9	-13
♀	63	71	261	-6	-8	-12	-28	1148	-11	-17	-21	-35
<i>(C) Severe diabetic</i>												
♂	27	66	107	+21	+46	+47	+61	758	-1	+20	+20	+61
♀	31	69	330	+1	+3	+5	+4	1363	-8	-4	-4	+7
♂	28	65	321	+4	+5	+6	+5	750	+22	+13	+93	+147

Dose = 2 g in all cases except as follows: * 3 g; † 1 g.

The average normal response to tolbutamide is shown in Fig. 1. In the 4 normal subjects blood glucose and NEFA reached a minimum approximately 40 minutes after injection. Thereafter the concentration of NEFA increased rapidly to a level above the control, while glucose returned more slowly toward the baseline value. Stable diabetics responding to tolbutamide showed a more protracted hypoglycemia with a parallel sustained reduction of NEFA concentration ($p = 0.003$) (Fig. 2).

Tolbutamide administration resulted in no decrease of blood glucose or NEFA in the group of severe diabetics (Fig. 3). Their response differed significantly ($p < 0.003$) from the effects seen in the combined groups of normal and mildly diabetic subjects. One patient, a juvenile diabetic in ketosis, had a marked elevation of both glucose and NEFA, which was unaffected by tolbutamide but responded promptly to insulin (Fig. 4).

Discussion. As indicated by the low p values, diabetics showed highly significant differences from normal in 2 respects: a prolonged fall of glucose and NEFA in mild diabetics, and absence of fall in the severe cases. Presumably these abnormal responses to tolbutamide were symptomatic of some metabolic disturbance in diabetes, but it must be

emphasized that the sample size was small, and conceivably the diabetics might have been atypical.

The findings support the current theory that tolbutamide stimulates insulin discharge, but does not in itself promote oxidation of glucose. In general it appears that NEFA level rises when carbohydrate is being utilized at a reduced rate, and falls when carbohydrate oxidation is enhanced. The relation probably reflects actual utilization of glucose, rather than availability of sugar, because glucose is ineffective in lowering NEFA level under conditions of insulin deficiency. With diabetic ketosis, and to a lesser extent with simple fasting, NEFA level rises progressively. Insulin causes a drop of NEFA in either case (8,9), whereas glucose is effective only in the fasting subjects. If given to severe diabetics without supplementary insulin, glucose adds to the hyperglycemia without affecting NEFA level.* Summing the evidence, it seems likely that tolbutamide acts to relieve ketosis only when the pancreas is capable of responding with an increased output of insulin.

Summary. Tolbutamide (Orinase), given intravenously to normal and diabetic patients

* Unpublished observations.

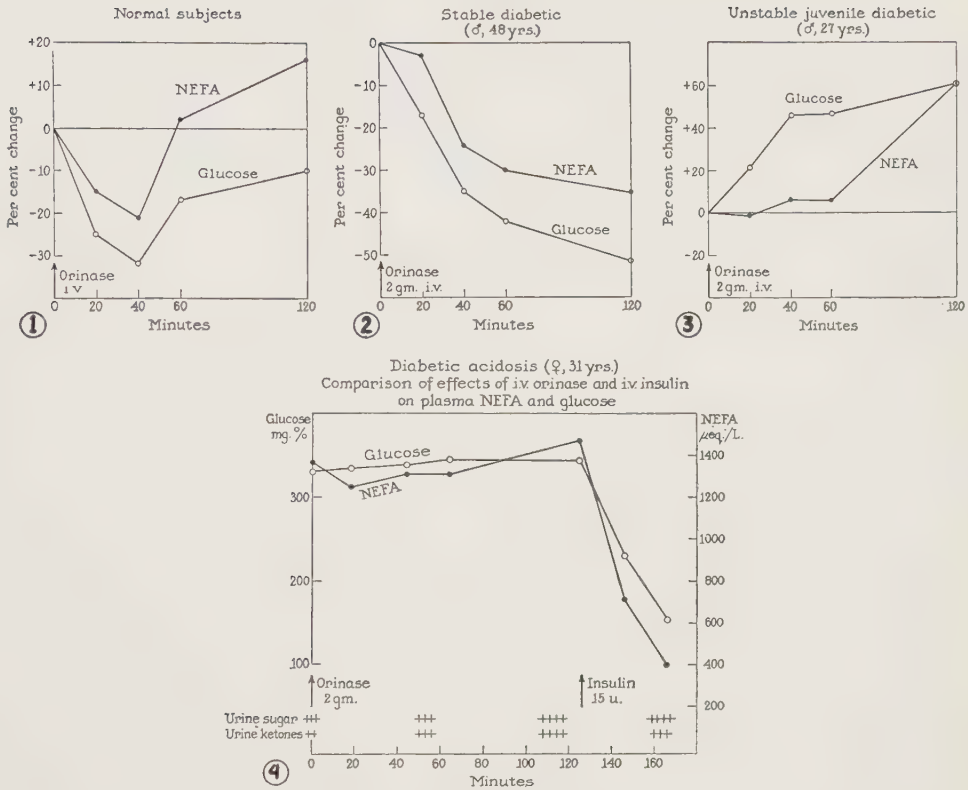


FIG. 1-4.

caused a reduction of non-esterified fatty acid concentration only in those subjects who responded with hypoglycemia. Diabetics responding to tolbutamide with a prolonged depression of blood sugar showed a comparably sustained reduction of the fatty acid fraction.

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Localization of Mycobacterial Enzymes.*† (23245)

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Schneider and Hogeboom(1) pointed out the need for quantitative studies in order to ascertain the true localization of enzymes in particulate and soluble fractions of mammalian cells. It is their opinion that enzymatic activity of isolated fractions should be compared quantitatively with total activity of the cells. Only from such a comparison can conclusions be drawn regarding intracellular distribution of enzymes. Two criteria often used for localization of enzymes are percent recovered activity and specific activity (activity per unit of nitrogen). If a high percentage of enzyme activity is recovered in a fraction and if concentration of enzyme (specific activity) is sufficiently high when compared with total cell activity then that fraction is considered the site of enzyme activity. The results of a quantitative enzymatic localization study of *Mycobacterium tuberculosis* var. *hominis* strain H37Ra have been presented(2). Terminal respiratory enzymes were localized either in a supernatant or one or more particulate fractions. In this investigation the criterion used for localization was dependent on the total quantity of enzyme recovered in a fraction. The fraction containing the highest activity of enzyme was considered the locus of this enzyme within the cell.

It is the purpose of this work to present results of quantitative studies of enzymes of the tricarboxylic acid cycle and of the terminal electron transport system in 5 strains of mycobacteria using the two criteria mentioned above. We hope to show that these criteria often lead to contradictory results which makes necessary some modification.

Materials and methods. This study was

carried out with the following organisms: *Mycobacterium tuberculosis* var. *hominis* strains H37Rv, H37Ra and H37RaN;‡ *Mycobacterium tuberculosis* var. *bovis* strain BCG-4;§ and *Mycobacterium smegmatis*. Throughout this paper the strains of *Mycobacterium tuberculosis* will be referred to by the abbreviations H37Rv, H37Ra, H37RaN and BCG. All organisms except *M. smegmatis* were grown as described previously(2). *M. smegmatis* was grown as a pellicle on Difco nutrient broth and harvested after 4 to 7 days incubation at 37°C by centrifugation and/or filtration through medium sintered glass filters. After harvesting, the cells were prepared and fractionated as reported previously(2). All precipitates were homogenized with a hand driven glass homogenizer to insure a homogeneous suspension, and the nitrogen content of each fraction was determined by the micro-Kjeldahl method of Ma and Zuzaga(3). The fractions were labeled L, A, B, C and S as previously reported(2). The L fraction contained particles approximately 100-200 m μ in diameter, the A contained particles approximately 50 m μ in diameter, the B contained particles approximately 25 m μ in diameter, the C contained particles approximately 19 m μ in diameter and the S represented supernatant after the removal of all particulate fractions. With fractions from H37Rv, BCG and *M. smegmatis* the presence of aconitase, fumarase, malic and succinic dehydrogenases was tested by the methods described by Colowick and Kaplan(4); catalase by the method of Lawrence and Halvorson(5); DPNH oxidase by the method of Green, Mackler *et al.*(6); isocitric dehydrogenase by the method of Hogeboom and Schneider(7); and alpha-ketoglutaric dehy-

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†† Medical Research Fellow, National Foundation for Infantile Paralysis.

‡ A mutant of *M. tuberculosis* var. *hominis* strain H37Rv transferred on a nitrogen deficient medium for over 6 years.

§ Kindly supplied by Dr. Sol Roy Rosenthal, Tice Clinic, University of Illinois, Chicago.

TABLE I. Localization of Cytochrome Oxidase in Mycobacteria.

	CE	Fraction					Total
		L	A	B	C	S	
Units of activity*							
H37Ra	4,742	1,697	693	173	31	1,175	
BCG	1,536	215	156	147	34	482	1,034
<i>M. smegmatis</i>	2,072	163	680	155	51	1,174	2,223
H37RaN	2,235	602	143				745
H37Rv	2,937		211	246	147	2,204	2,808
% CE							
H37Ra	(100)	35.8	14.6	3.7	.7	24.8	79.6
BCG	"	14.0	10.2	9.6	2.2	31.4	67.4
<i>M. smegmatis</i>	"	7.9	32.8	7.5	2.5	56.7	107.4
H37RaN	"	27.0	6.4				33.4
H37Rv	"		7.2	8.4	5.0	75.0	95.6
Specific activity							
H37Ra	(1)	2.41	4.76	1.75	1.03	.50	
BCG	"	2.23	2.08	6.33	2.38	.39	
<i>M. smegmatis</i>	"	1.94	9.24	3.90	2.50	.87	
H37RaN	"	2.34	1.08				
H37Rv	"		1.57	1.92	1.74	1.03	

* 1 unit of enzyme is that amt of fraction causing uptake of 1 μ l O₂ in 1 hr.

drogenase by the method of Lindstrom(8). Cytochrome oxidase was determined by the method described previously(2). With fractions from H37RaN and H37Rv a modified Thunberg technic was used for determining malic, isocitric and alpha-ketoglutaric dehydrogenases. This was necessary because of the virulence of H37Rv and the potential virulence of H37RaN. Eight ml screw cap tubes were employed. To each tube was added 1.6 ml of 0.5M phosphate buffer pH 7.2, 0.1 mg methylene blue, 1 ml of 0.2M substrate adjusted to pH 7.2 with KOH, and fractions. When testing for isocitric dehy-

drogenase 5 μ moles of MgSO₄; 5 μ moles of MnCl₂ and 1 mg TPN^{||} were added. When testing for malic dehydrogenase 5 μ moles of MgSO₄ and 1 mg DPN[¶] were added. When testing for alpha-ketoglutaric dehydrogenase 5 μ moles of MgSO₄, 5 μ moles of MnCl₂, 1 mg DPN, and 0.2 mg Co A concentrate (Armour, 13.0 Lipmann units/mg) were added. The final volume of each tube was made up to 8 ml with distilled water and the tubes were then incubated in a 37°C water bath. Rate of reduction of methylene blue was observed and recorded. One unit of the enzyme was that amount of fraction causing the trans-

TABLE II. Localization of DPNH Oxidase in Mycobacteria.

	CE	Fraction					Total
		L	A	B	C	S	
Units of activity*							
H37Ra	24,100	7,360	3,180	348	84	1,100	12,072
BCG	1,670	920	897				1,817
% CE							
H37Ra	(100)	30.5	13.2	1.4	.4	4.6	50.0
BCG	"	55.0	53.7				108.8
Specific activity							
H37Ra	(1)	11.47	6.38	2.21	.89	.13	
BCG	"	4.00	6.55				

* 1 unit of enzyme is that amt of fraction causing decrease of 0.001 optical density unit in 1 min.

^{||} TPN: triphosphopyridine nucleotide.

[¶] DPN: diphosphopyridine nucleotide

TABLE III. Localization of Succinic Dehydrogenase in Mycobacteria.

	CE	Fraction			Total
		L	A	B	
Units of activity*					
H37Ra	76	53	14		67
BCG	61	21		1.5	22.5
<i>M. smegmatis</i>	2,627	1,302	1,449		2,751
H37RaN	306	66	46		132
H37Rv	68	14	25	16	55
% CE					
H37Ra	(100)	69.7	18.4		88.1
BCG	"	34.4		2.5	36.9
<i>M. smegmatis</i>	"	49.6	55.2		104.8
H37RaN	"	21.6	15.0		36.6
H37Rv	"	21.0	36.7	23.5	81.2
Specific activity					
H37Ra	(1)	4.33	5.32		
BCG	"	4.01		1.35	
<i>M. smegmatis</i>	"	80.71	13.67		
H37RaN	"	1.89	2.28		
H37Rv	"	5.10	2.78	2.51	

* 1 unit of enzyme is that amt of fraction causing a transfer of 1 μ l H₂ to methylene blue in 1 hr.

fer of 1 μ l of hydrogen to methylene blue/hour. The localization of fumarase, aconitase, and DPNH was not determined. When an enzyme could not be localized by the methods described, the crude extract was tested for its presence by the standard Warburg manometric procedure before concluding that no activity was present. To the Warburg reaction vessels were added 5 μ moles each of MgSO₄ and MnCl₂, 0.1 mg DPN (or TPN in the case of isocitric dehydrogenase), 0.1 mg ATP,** 0.5 ml of 0.1M substrate adjusted to pH 7.2 with KOH and 0.1 ml of the crude extract. To the center well was added 0.2 ml of 20.0% KOH. In each case the endogenous respiration was measured and subtracted from that of the test vessel.

Results. Tables I-III and Figs. 1-6 show the results of enzyme localization studies with 5 mycobacteria. These results are reported both as percent of recovered activity (the percent of activity in a fraction compared with that found in the unfractionated crude extract) and as specific activity (activity per mg nitrogen). Specific activities of one or more were considered significant since this represented either the same concentration or

a greater concentration of enzyme found in the crude extract (arbitrarily assigned a value of 1.0).

With fractions of H37Ra, BCG, *M. smegmatis*, and H37RaN both criteria indicate that cytochrome oxidase activity is localized in the particulate fractions (Table I). A high percentage of recovered activity was found in the particulate fractions and these fractions possessed significant (above 1.0) specific activities. With strain H37Rv this enzyme appeared to be localized in the supernatant fraction which possessed 75% of recovered activity and in addition a significant specific activity. Although the supernatant fraction of all strains tested possessed a high percentage of recovered activity this fraction of H37Rv only possessed a significant specific activity.

Table II shows the results of localization studies of DPNH oxidase with strains H37Ra and BCG. There was good agreement between the 2 criteria for localization. This enzyme, by both criteria, was localized in the 2 larger particulate fractions L and A.

The results with succinic dehydrogenase (Table III) again demonstrated good agreement between the 2 localization criteria. This enzyme was localized in the particulate fractions L and A, or L, A and B of all strains tested. With strains BCG and H37RaN rather poor total recoveries were obtained (36.1 and 36.6% respectively). However, the high concentration of enzyme activity in the particulate fractions makes the presence of undetectable enzyme in the supernatant fractions doubtful.

Figs. 1-6 show that aconitase, catalase, fumarase, isocitric, alpha-ketoglutaric and malic dehydrogenases could not be localized because of the lack of correlation between the 2 criteria used. In many cases where the supernatant fractions possessed the highest % of recovered activity they did not possess significant specific activities. On the other hand, many fractions possessed significant specific activities and very low or insignificant percentages of recovered activity. Further complicating the localization attempt was the fact that some fractions which pos-

** ATP: adenosine triphosphate.

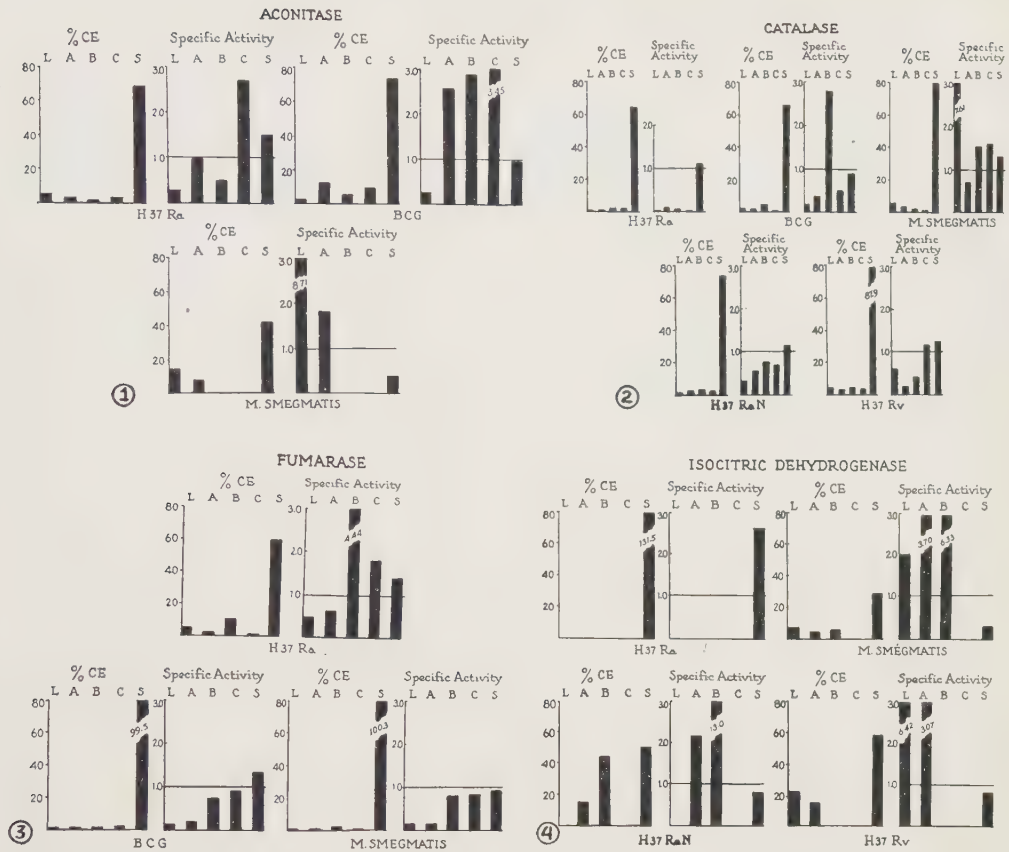


FIG. 1 (top left). Comparison of percent recovered activity (% CE) and specific activity of aconitase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 2 (top right). Comparison of percent recovered activity (% CE) and specific activity of catalase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 3 (bottom left). Comparison of percent recovered activity (% CE) and specific activity of fumarase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 4 (bottom right). Comparison of percent recovered activity (% CE) and specific activity of isocitric dehydrogenase. Specific activities less than 1 (horizontal line) are considered insignificant.

sessed the highest percentage of recovered activity and a significant (above 1.0) specific activity often possessed lower specific activities than other fractions of the same organism with insignificant percentages of recovered activity.

Isocitric, alpha-ketoglutaric and malic dehydrogenases could not always be found in fractions of all organisms tested although often positive Warburg manometric tests of the crude extract proved their presence. Isocitric dehydrogenase (Fig. 4) could not be found in extracts of BCG whereas the crude extract showed an average of 88 μ l of

oxygen uptake over that of the endogenous. Alpha-ketoglutaric dehydrogenase (Fig. 5) could not be localized in fractions of either BCG or *M. smegmatis* whereas the crude extract of only *M. smegmatis* showed an uptake of 16 μ l/ml over that of the endogenous. Malic dehydrogenase (Fig. 6) could not be found in fractions of *M. smegmatis* and manometric tests of the crude extract produced negative results.

One possible explanation for the lack of correlation between the 2 criteria and the fact that catalase, fumarase, isocitric dehydrogenase, alpha-ketoglutaric dehydrogenase and

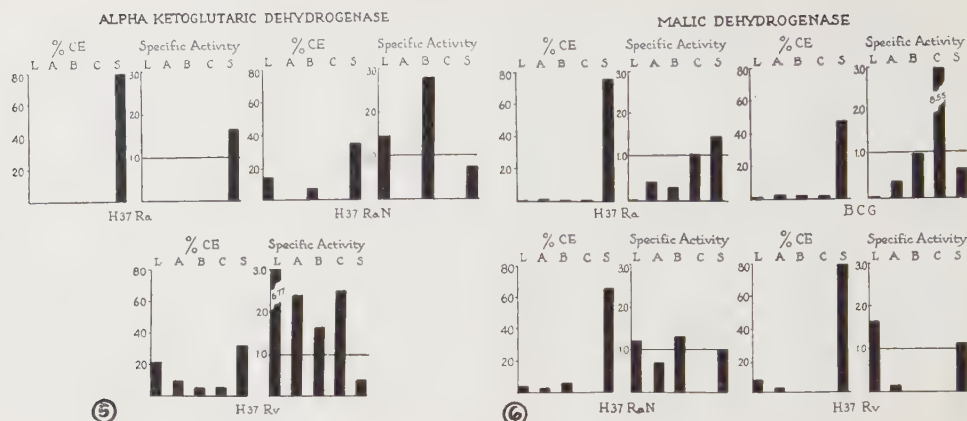


FIG. 5 (left). Comparison of percent recovered activity (% CE) and specific activity of alpha-ketoglutaric dehydrogenase. Specific activities less than 1 (horizontal line) are considered insignificant.

FIG. 6 (right). Comparison of percent recovered activity (% CE) and specific activity of malic dehydrogenase. Specific activities less than 1 (horizontal line) are considered insignificant.

malic dehydrogenase could not be definitely localized in either particulate or supernatant fractions could be that soluble enzymes from the supernatant fractions became adsorbed to the particles of the particulate fractions during preparation. If the major portion of the particulate nitrogen could be attributed to

adsorbed enzymes then this could account for high specific activities along with low % recoveries in many of the particulate fractions. To allow for this possibility modifications in criteria were made. An arbitrary figure of 10% was adopted as the maximum amount of enzyme that might be adsorbed from the su-

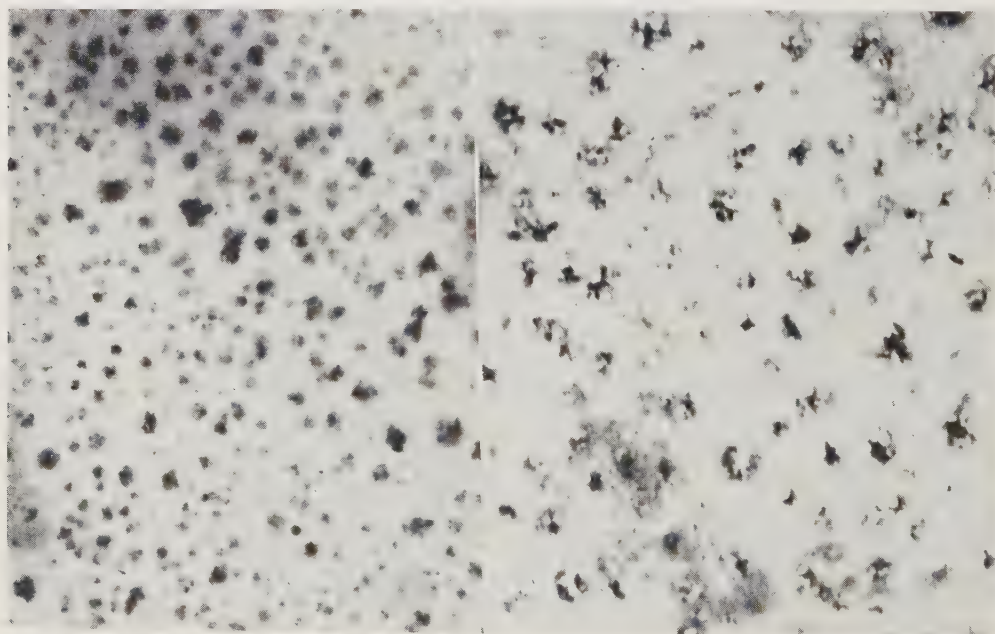


FIG. 7a (left). Crude extract of *Mycobacterium tuberculosis* var. *hominis* strain H37Ra showing aggregates of varying sized particles. $\times 5080$.

FIG. 7b (right). Same field as in Fig. 7a after 45 sec. exposure to electron beam. $\times 8620$.

pernatant under the conditions of our fractionation scheme. Criteria were modified therefore, so that only fractions containing 10% of recovered activity or higher and at the same time specific activities of 1.0 or greater were accepted as possible enzyme loci.

Using this new criterion it was found that most of the tricarboxylic acid cycle and terminal electron transport enzymes could be localized in one or more fractions of the strains of mycobacteria tested. The exceptions were catalase, and malic dehydrogenase in BCG and fumarase and isocitric dehydrogenase in *M. smegmatis*. In all probability fumarase is localized in the supernatant fraction of *M. smegmatis* as was true with the other organisms tested since this fraction of *M. smegmatis* possessed not only most of the total recovered activity but a specific activity of 0.93. The total recovery of isocitric dehydrogenase from fractions of *M. smegmatis* was poor (47.2%) which might explain the failure to localize this enzyme. We can find no explanation for the failure to localize catalase in fractions of BCG.

Discussion. It becomes apparent from the results that the fraction showing the greatest percentage of recovery does not always coincide with that showing the greatest concentration of specific activity. It seemed logical to expect a fraction to contain at least 10% of enzyme activity in order to be accepted as a possible locale for this enzyme. A specific enzyme activity of 1.0 or greater should also be expected because the consideration of only those fractions which contain greatest percentages of recovered activity as enzyme loci automatically eliminates the possibility of multiple loci. A single locus appears unlikely since several fractions possessed moderate percentages of recovered activity for a single enzyme. Furthermore, eliminating fractions with less than 10% of recovered activity from consideration as enzyme loci would more than compensate for adsorption as a source of error. Therefore, the finding of at least 10% of recovered activity with a specific activity of 1.0 or more in a given fraction was adopted as a new criterion for enzyme localization.

The data presented suggest the possibility of the presence of true mitochondria in mem-

bers of the family Mycobacteriaceae. The isolated particles contain enzymes of terminal electron transport as well as enzymes concerned with oxidative metabolism. Of prime interest is the fact that these particles were opaque and labile to electron bombardment. The crude extract contained a mixture of varying sized particles (Fig. 7A). The lability of the particles was evident by volatilization after 45 seconds of exposure (Fig. 7B). Mudd, Takeya *et al.*(9) recently showed the existence of rosettes of particles within BCG cells which could also be volatilized by the electron beam. These intracellular particles were of approximately the same size range as those reported here. It is entirely possible that the entire rosette reported by these workers represents the particles in our L fraction and that the particles in the A, B and C fractions represent fragments of larger particles. This fragmentation could also explain the presence of high concentrations of enzymes such as cytochrome oxidase and DPNH oxidase in the supernatant fractions. These enzymes may have been solubilized by the drastic grinding procedure necessary to break up the relatively sturdy mycobacterial cells.

The localization results are in rather close agreement with those of several other workers. Yamamura, Kusunose *et al.*(10) and Kusunose, Nagai *et al.*(11) found that succinic dehydrogenase and DPNH oxidase along with the cytochromes resided in the particulate fraction of *Mycobacterium avium*. Their fraction would probably correspond to the L and A fractions combined. They reported, however, that malic dehydrogenase was localized in the particulate fraction while with the organisms studied here it was localized in the supernatant fraction. Alexander and Wilson's(12a, b) data are also quite similar to those reported here. They reported that cytochrome oxidase, DPNH oxidase and succinic dehydrogenase were localized in the particulate fractions of *Azobacter vinelandii*. Catalase, aconitase, isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase were localized in the supernatant fraction. Their data concerning localization of a malic acid oxidizing enzyme agreed with Yamamura and

his co-workers since malic oxidase was localized in the particulate fractions.

The only differences between strains which might be directly or indirectly concerned with virulence were found in the localization of cytochrome oxidase, isocitric dehydrogenase and alpha-ketoglutaric dehydrogenase. Cytochrome oxidase appeared to be soluble with the virulent H37Rv strain and particulate bound with the other strains tested. Isocitric and alpha-ketoglutaric dehydrogenases appeared to be particulate bound with the virulent H37Rv and its mutant H37RaN strain but soluble with the other strains tested. The significance of these differences must await further studies.

Summary. *Mycobacterium tuberculosis* var. *hominis* strains H37Rv, H37Ra and H37RaN; *Mycobacterium tuberculosis* var. *bovis* strain BCG-4; and *Mycobacterium smegmatis* were ground in a ball mill and fractionated according to the methods outlined earlier(2). Enzymes aconitase, fumarase, DPNH oxidase, catalase, cytochrome oxidase, isocitric, alpha-ketoglutaric, malic and succinic dehydrogenase were localized in one or more fractions using the two criteria established by Alexander and Wilson(12a, b). Cytochrome oxidase, DPNH oxidase and succinic dehydrogenase were found to be particulate bound by both criteria. However, the locus of other enzymes determined by one criterion did not always coincide with that determined by the second criterion. A modified criterion was then established and this was discussed. With the new criterion most of the

enzymes of the tricarboxylic acid cycle and terminal electron transport system could be localized. The particles found within the various fractions were electron opaque and labile to electron bombardment. The presence of enzymatic activity, particularly those enzymatic activities concerned with energy yielding mechanisms lends support to the hypothesis that these particles are mitochondria.

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Host Resistance in Hemorrhagic Shock: VIII. Effect of Properdin on *in vitro* Function of Phagocytes.* (23246)

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Hemorrhagic shock in the dog lowers the Properdin titer in blood to or near zero within

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a few hours(1). Another effect of hemorrhagic shock is a fall in phagocytic and bacteriostatic indexes of phagocytes, which has been attributed to the presence of a leukotoxin in the plasma(2). Recently a lethal

TABLE I. Effect of Properdin on Functional Activity of Phagocytes in Normal and Shock Plasma.*

	Granulocytes				Macrophages			
	N.P.	N.P. + P.	S.P.	S.P. + P.	N.P.	N.P. + P.	S.P.	S.P. + P.
Phagocytic index	49 ± 10†	48 ± 8	21 ± 5	19 ± 7	44 ± 8	46 ± 8	23 ± 5	26 ± 5
Bacteriostatic index	69 ± 9	69 ± 11	20 ± 5	22 ± 7	41 ± 8	39 ± 4	21 ± 6	28 ± 8

* Each figure is mean of 4 or more experiments.

† ± stand. dev.

N.P., normal plasma; P., Properdin; S.P., shock plasma.

toxin, in addition to a leukotoxin, has been identified in blood of hemorrhagic shock.† Whether either toxin is a bacterial endotoxin, or a polysaccharide derived from host's tissues(3), it is possible that the fall in titer of Properdin in shock plasma might be due to binding to toxin. Although the depression of the phagocytic and bacteriostatic activity of phagocytes is presumably caused by the toxin, it is possible that it might be caused by a deficiency of Properdin. In either case one might expect to restore the function of these cells to normal by providing an excess of Properdin. This was investigated by *in vitro* studies of the effect of added Properdin upon phagocytic and bacteriostatic activity of phagocytes in plasma of normal and shocked animals.

Method. Macrophages and granulocytes were harvested from peritoneal cavities of normal rabbits, and from peritoneal cavities of rabbits 6 hours after intraperitoneal injection of beef infusion broth, by a method described elsewhere(4). Plasma was obtained from normal rabbits, and from rabbits in advanced hemorrhagic shock following failure to respond to transfusion. The following mixtures of plasma (0.2 ml) and cells (10 million cells in 0.1 ml of gelatin-Locke's solution) were prepared in duplicate: granulocytes in normal plasma; granulocytes in shock plasma; macrophages in normal plasma; and macrophages in shock plasma. To each mixture 0.01 ml of a 5 hour culture of Friedlander's bacillus was added, and the phagocytic and bacteriostatic activity of the cells determined by a technic described elsewhere(2). The same tests were run simultaneously in duplicate, using plasma fortified with Properdin‡

added to yield a concentration of 32 u/ml.

Results. Table I lists the phagocytic and bacteriostatic indexes of granulocytes and macrophages in normal and shock plasma, and in the same plasmas with Properdin added. In accordance with Pillemer's findings,§ Properdin had no effect on phagocytosis. This is true for both types of cell, and in both normal and shock plasma. The same results apply to bacteriostasis.

To eliminate the possibility that an excess of Properdin might inhibit a salutary effect of this substance, several determinations were performed using only 8 units of Properdin/ml of plasma. The results were the same as with the higher concentration of Properdin. Several experiments were done with another test organism (*E. coli*). In this case, too, Properdin did not alter the phagocytic or bacteriostatic activity of the cells.

The depression in phagocytic and bacteriostatic activity of the phagocytes by shock plasma can be eliminated by diluting the shock plasma with normal plasma. The data in Table II show that Properdin added to progressive dilutions of shock plasma with normal plasma does not materially influence the phagocytic or bacteriostatic index of the cells.

Conclusion. The phagocytic and bacteriostatic activity of phagocytes *in vitro* is not influenced by the titer of Properdin in plasma

† Schweinburg, F. B., Shapiro, P., Fine, E., and Fine, J., PROC. SOC. EXP. BIOL. AND MED., in press.

‡ Properdin, complement and magnesium ions constitute the components of the Properdin system(5). It has been previously demonstrated that complement does not fall in hemorrhagic shock(6). Although no magnesium determinations were performed, significant electrolyte imbalance does not develop in simple hemorrhagic shock produced by the technic we employ.

§ Personal communication.

TABLE II. Effect of Normal Serum with and without Properdin on Functional Activity of Granulocytes in Shock Plasma.

N.P. added to S.P.		Phagocytic index (Mean of 4 exp.)	Bacteriostatic index (Mean of 4 exp.)
.00	.2	15 ± 3	24 ± 7
.01	.19	15 ± 3	20 ± 8
.02	.18	18 ± 6	31 ± 14
.04	.16	27 ± 3	31 ± 14
.08	.12	39 ± 8	43 ± 15
.16	.04	44 ± 8	68 ± 19
.20	.00	49 ± 7	68 ± 12

N.P. + P. added to S.P.		Phagocytic index (Mean of 4 exp.)	Bacteriostatic index (Mean of 4 exp.)
.00	.2	15 ± 3	24 ± 7
.01	.19	16 ± 4	31 ± 9
.02	.18	21 ± 2	33 ± 8
.04	.16	31 ± 8	51 ± 9
.08	.12	39 ± 5	73 ± 8
.16	.04	45 ± 8	69 ± 9
.20	.00	49 ± 7	68 ± 12

of normal or shocked animals.

We are grateful to Dr. Louis Pillemer for the Properdin, which was stored at -20°C until immediately before use.

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Failure to Transmit Radiation-Induced Early Hypotension in Rabbits Connected Through Controlled Cross-Transfusion.* (23247)

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In rabbits, whole-body x-irradiation with doses above 50 r induces a blood pressure fall that becomes evident within 2 hours after exposure(1,2). Some experimental findings suggest that a similar hypotensive reaction may occur in a normal test animal after injection of blood originating from an irradiated rabbit(1). Is the blood pressure drop really caused by a blood-borne chemical factor? A decisive answer may be expected from continuous cross-transfusion studies. This type of experiment requires a high rate of blood exchange throughout a period of several hours, and conservation of a stable mean blood volume in each of the connected animals, even though a difference in blood pressure may develop. The present report describes a blood pump that fulfills the requirements and some

results obtained with the equipment.

Methods. General procedure. With the arrangement depicted in Fig. 1, cross-transfusion was started at 30 min prior to irradiation, continued throughout the exposure time of 30 min, and concluded approximately 3 hours after irradiation. Then the blood volume of each rabbit was determined by the Evans blue method and the body weight remeasured. During transfusion the sequence of events was as follows: (I) withdrawal of blood from first animal, (II) change of valve position, (III) injection into second animal, (IV) withdrawal of blood from second animal, (V) change of valve position, (VI) injection into first animal; thereafter the next cycle started again with (I). In the present experiments motor speed was so regulated as to furnish three complete cycles every minute. Dead space in polyethylene tubing and valve amounted to 1/40 cc; therefore, a stroke vol-

* The author is indebted to H. A. Jaeger who constructed the cross-transfusion pump in the USAF School of Aviation Medicine.

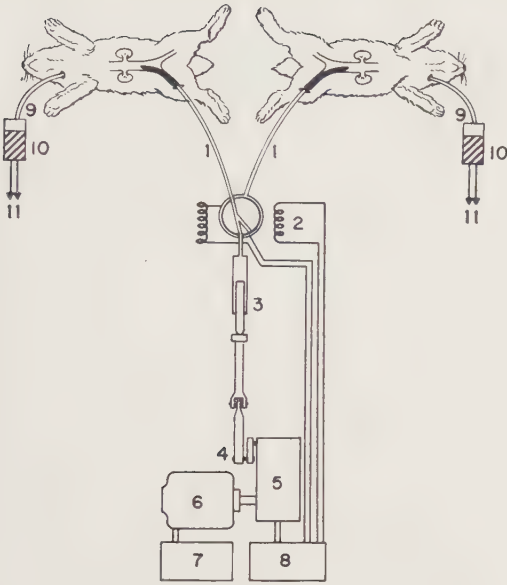


FIG. 1. Schematic drawing of experimental arrangement, the numbered sections of which represent: (1) polyethylene catheters introduced through femoral arteries, the tips are located in lower abdominal aorta halfway between iliac bifurcation and origin of renal arteries; (2) nylon valve with 2 rotary relays controlling its position; (3) 2 cc Luer syringe; (4) crank with adjustment for stroke volume of syringe; (5) box containing 853:6 gear ratio and 2 micro-switches operated by cams that are driven by the gear assembly; (6) 1/15-horsepower motor; (7) speed regulator for motor; (8) power supply box containing rectifier and 2 relays that, when triggered by the micro-switches in (5), energize the rotary relays in (2) and, thereby, control valve position; (9) polyethylene tubings in carotid arteries; (10) strain gauge for blood pressure recording; (11) connections to Heiland oscillograph recorder.

ume of slightly more than 2 cc yielded an effective volume of 2 cc, or an exchange rate of 6 cc/min/animal. For each rabbit the amount (c) of own blood in percent of its blood volume (v), as a function of the number (n) of pump cycles performed with an effective displacement (s), could be calculated from the

$$\text{equation } c = 50 \left(1 + e^{-\frac{2sn}{v+s}} \right).$$

Under the present experimental conditions (s, 2 cc; v, approximately 160 cc; and n, 3/min) a 55/45 mixture of bloods was reached within 30 min.

Irradiation. One animal of each pair was well shielded by a lead cover, while the other partner received whole-body x-irradiation under the following conditions: 260 kvp, 18

ma; inherent filtration, 0.25 mm copper; additional filtration, 1 mm aluminum and 0.5 mm copper; half-value layer, 1.4 mm copper; dose rate, 30 r/min.; exposure time, 30 min.; and total dose at center of animal, approximately 900 r, as measured in air with a Victoreen ionization chamber.

Results. Ten final experiments yielded uniform and convincing results. In all instances the procedure was well tolerated. Even after 5 hours of continuous cross-transfusion, hemolysis could not be detected and the difference in body weights between the two partners did not exceed initial value by more than 10 g, indicating a shift in blood volume of less than 10 cc. The shielded partners revealed blood pressure records that corresponded essentially to those obtained from nonirradiated single control animals. Thus it must be concluded that a damaging chemical factor, if existing in the blood of the irradiated rabbit, was not transferred in concentration sufficient to depress pressure in the nonirradiated partner. The irradiated animals showed tracings that corresponded closely to those obtained from single rabbits exposed to the same dose. All exhibited a pressure fall, reaching critical values within 1 to 2 hours following start of exposure (Fig. 2). Time course, degree, and final outcome of the hypotensive reaction seemed to be alike for cross-transfused and single rabbits. Thus it must be concluded that a beneficial chemical

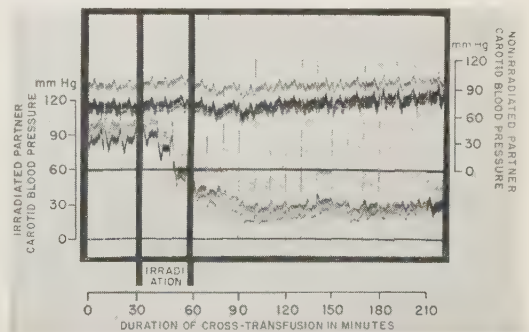


FIG. 2. Sections, covering 1 min. each, cut at 10-min. intervals from continuous carotid blood pressure records of 2 rabbits connected through cross-transfusion. During the 30 min., represented by the 3 sections between the two heavy bars, the animal of the lower tracing received 900 r whole-body x-irradiation, while that of the upper tracing was well shielded.

factor, if existing in the blood of the non-irradiated animal, was not transferred in concentration sufficient to ameliorate the pressure drop in the irradiated partner.

Discussion. Results of the present experiments leave no doubt that irradiated and non-irradiated rabbits connected through cross-transfusion behave like independent animals so far as blood pressure is concerned. Although absence of a vaso-depressive factor in the arterial blood does not exclude its existence at the tissue level and/or in the venous blood, the findings point to the nervous system as an important participant in the early hypotensive reaction. This view is supported by the facts that atropinization and vagotomy strongly reduce the initial blood pressure response in rabbits(1,3), and that doses between 1500 and 2500 r cause hypotension in normal but not in spinal rats(4).

Summary. A pump for continuous cross-transfusion has been developed, and some studies about the nature of the early blood pressure fall induced in rabbits by x-irradiation have been conducted. Results of the cross-transfusion experiments indicate that the shielded animal does not protect its partner exposed to 900 r, neither does the irradiated rabbit affect the blood pressure of the shielded partner.

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Method for Temporary Exclusion of Liver in Dogs.* (23248)

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The major causes of the mortality associated with a one-stage hepatectomy in dogs are hemorrhage and air embolization. Leveen and Lewis(1), by substituting a plasticized vinyl tubing for the glass cannula originally described by Markowitz(2), greatly increased the number of surgical survivals. The liver is a unique organ in at least two respects. First, it has a double blood supply, the hepatic artery and portal vein. Occlusion of the latter results in rapid death, because the inflow of blood into the intestinal area has no return outlet to the general circulation. This loss of effective circulating volume results in irreversible shock. The liver is also unusual in dogs in that it drains directly into the vena cava without benefit of intervening blood vessels. In order to perform a successful total hepatectomy, therefore, the portal blood must be short-circuited into the

vena caval (systemic) system, and a method must be utilized to block the venous outflow from the liver without occluding the vena cava.

Method. By reference to the diagram (Fig. 1) one can see how both these problems are met rather simply by the plastic cannula. A portal-caval anastomosis is established merely by insertion of the side arm of the tube into the distal divided portal vein. The hepatic venous drainage is trapped or occluded between ligatures A and B, while the systemic circulation can proceed unimpeded by the vena cava through the central portion of the tube. If the arterial supply is satisfactorily controlled the liver can now be removed with minimal blood loss and no compromise or block of the animal's circulation. If one desires, however, to reverse the hepatectomy 2 modifications are necessary. The artery must be occluded by means of a

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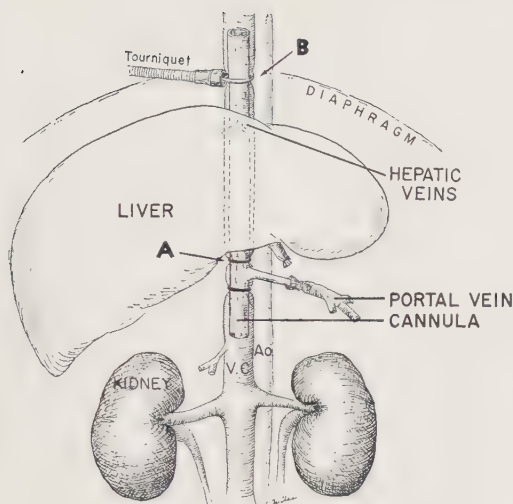


FIG. 1.

non-crushing clamp, rather than divided, so that its integrity can be easily restored. The ligature compressing the vena cava against the tube between the liver and diaphragm should be replaced by a tourniquet; its release immediately reestablishes hepatic out-

flow. The portal circulation, too, may be restored rapidly by interposing a tube between the divided vein ends(3). This avoids the necessity of a suture anastomosis and can be carried out with great facility. All tubes used for vascular anastomosis must be suitably treated with Desicote to prevent vascular thrombosis. With experience, the mortality of this "reversible hepatectomy" should not exceed 15%.

Summary. An experimental technic has been described by which the liver can be excluded from the dog's circulation and returned promptly, as desired, into its normal anatomical and functional position. This method has a low mortality.

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Uptake of Fission Products and Neutron-Induced Radionuclides by the Clam. (23249)

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(Introduced by E. M. Jacobsen)

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Detection of sea water contamination resulting from radioactive fallout or disposal of reactor waste products deserves careful consideration(1,2). Assessment of degree of contamination of water in which levels of activity are low, but nevertheless biologically significant, is difficult owing to the large volume of water required for radioanalysis. A biological indicator which rapidly reflects degree of nuclide contamination by virtue of a concentrating ability is of obvious value. Surveys made of the Marshall Islands one and 2 years after their accidental contamination by fallout, revealed the presence of readily detectable levels of radioactivity in clams (3,4). Radioactivity in these animals was

significantly higher per unit body weight than in most marine specimens analyzed. An extraordinary ability to concentrate radionuclides dispersed in sea water was evident from the fact that the level of gamma activity in clams collected at Eniwetok Proving Ground was about 2000 times greater than that of sea water(3). The concentrating ability of the clam probably derives from its mechanism of feeding which involves filtration of large volumes of water. It is estimated that in one year a 3 in. clam filters about 2.3×10^4 liters of water(5).

In the current study, the uptake by the clam of radionuclides was examined under controlled conditions. Groups of clams were

placed in sea water which contained mixed fission products, Zn^{65} , Co^{60} or Fe^{59} and uptake was measured at various intervals. Zn^{65} , Fe^{59} , and Co^{60} , all of which are neutron-induced activities,* were included in the study since at least 2 of these. Zn^{65} and Co^{60} , were detected in marine animals one and 2 years following exposure to fallout(3,4,6).

Procedure. Japanese littleneck clams averaging 2 in. diameter and weighing about 17 g, obtained from San Francisco Bay were used. A 2-month-old mixture of fission products† and the neutron induced activities Zn^{65} , Fe^{59} , and Co^{60} were each studied separately for each series. Four clams were placed into each aquarium containing one liter of sea water. A total of 80 clams were placed in 4 series of 5 aquaria, all of which were continuously aerated. To each of the 5 aquaria in a series, 5.2 μC Fe^{59} , 1.1 μC Zn^{65} , 5.4 μC Co^{60} , or approximately 5 μC mixed fission products was added. The radionuclides, with the exception of Fe^{59} , were of high specific activity. Clams in each series were killed at intervals of 3 hr, 6 hr, 1, 3 and 6 days. In another experiment, uptake of Co^{60} by clams was studied as a function of concentration. Four clams were placed into each of 3 aquaria which contained 0.9, 5.2, or 280 $\mu\text{C}/\text{l}$ of Co^{60} . These clams were killed on the sixth day. (1 μC of these solutions contained 0.23 mg of inert cobalt.) At termination of this experiment, the clams were weighed, the shells removed. Weight of shell and of soft tissue were then determined. Both shell and soft tissue were dried at 100°C and ashed at 550°C overnight. The ash was dissolved in HCl and diluted to volume. The gamma activity for neutron-induced activities in an aliquot was measured in a well-type sodium iodide scintillation detector and scaler. In addition, residual gamma activity in the water was determined at each sacrifice interval. The clams placed in the mixed fission product solution were analyzed radiochemically for Sr^{89} , Zr^{95} , $\text{Ru}^{103-106}$, and the rare

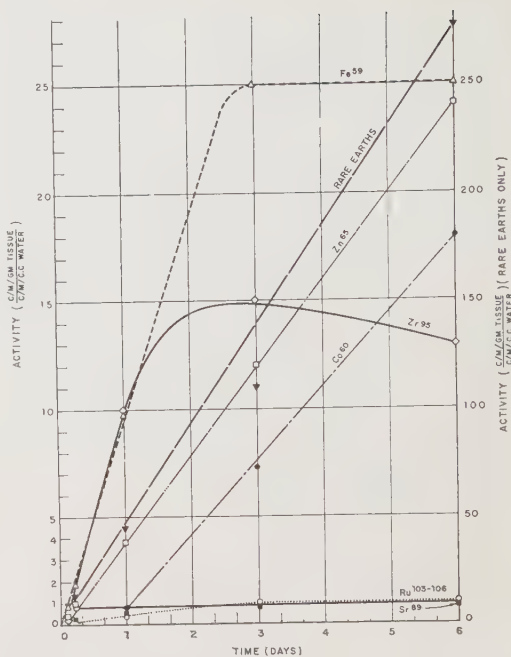


FIG. 1. Concentration ratios of radionuclides in soft tissue of clams.

earth group, as previously described(7). The water in the aquarium was also analyzed radiochemically to determine concentration of these same radionuclides.

Results. The results demonstrate that clams are able to take up and retain various radionuclides in both shell and soft tissue. The activity of the various radionuclides in the soft tissue of clams placed in radioactive solutions is shown in Fig. 1. Values are expressed in terms of a concentration ratio (ratio activity per gram soft tissue to activity per ml radioactive solution). This mode of expression was used because concentration of the radionuclide in the medium decreased significantly with time.

In general, much higher levels of activity were observed in the shells than in the soft tissues (Fig. 2). Rates of uptake by the shell of Fe^{59} , Zn^{65} , and Co^{60} are constant in the period from 1 to 6 days (Fig. 2). The concentration ratio for Co^{60} was 10-fold higher than for Zn^{65} and Fe^{59} . Studies performed with shells detached from the animal indicated that the mechanism of retention of the activity by the shell was a nonmetabolic process, since the activity was the same as

* Neutron-induced activities are radionuclides formed by capture of a neutron (released in nuclear fission) by the nucleus of a stable element.

† This fission product mixture was derived from fallout collected from a nuclear detonation.

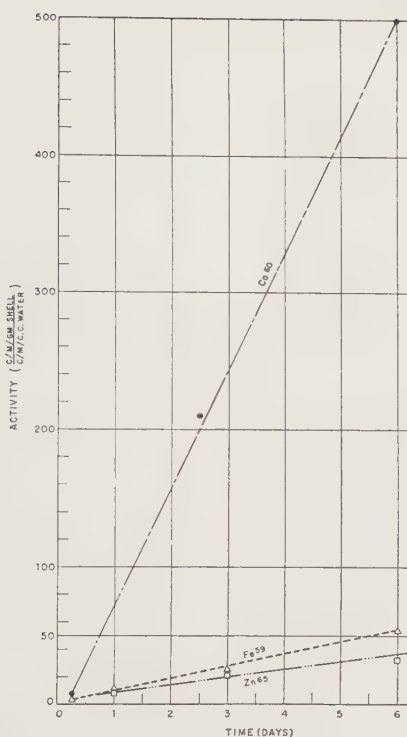


FIG. 2. Concentration ratio of radionuclides in shells of clams.

that of the shell of the living organism. While the uptake of activity by detached shells therefore was probably a surface adsorption phenomenon, the uptake by shells of living animals probably also involves a metabolic process(8). Incorporation of radionuclides into the soft tissues appears to be a metabolic process.†

The uptake of Co^{60} , the rare earth group, and Zn^{65} by the soft tissues was a linear function in the 1 to 6 day period, and similarly, a linear relation was observed for iron uptake during the first 3 days. From 3 to 6 days, however, concentration of Fe in the soft tissues remains constant, perhaps indicating a saturation condition with respect to this element. The low specific activity of the Fe^{59} probably accounts for this early saturation. Of the radionuclides studied, Fe was taken up at the highest rate, while Zn and Co were taken up at somewhat lower rates. The concentration ratio for the rare earths was 10-

fold greater than for any other radionuclide. Rate of uptake of Zr^{95} by the soft tissue was linear for the first day only and leveled off in the period from 2 to 6 days. The values measured during the first day indicate that the clam in most cases was in the process of attaining an equilibrium condition with the new environment. It was noted, for example, that not all the clams began to pump water at the same time after being transferred to the radioactive solutions.

Concentration ratios (activity/g tissue/activity per ml solution) of Co^{60} , Zn^{65} , and Fe^{59} in the soft tissues of the clam at 6 days varied from 18 to 25 (Fig. 1). This close agreement in concentrating ability probably reflects the rather similar chemical properties of these transition elements, which provide a basis for their common metabolic behavior.

Concentration ratios for mixed fission products at 6 days ranged from 1 for $\text{Ru}^{103-106}$ and Sr^{80} to 13 for Zr^{95} and 280 for the rare earth elements. Ru and Sr soon attain a concentration ratio of 1 and maintain this over the 6-day period, indicating that they are in equilibrium with, and equal to, the concentration of the solution.

Strontium appears in the tissues of mollusks in the same order of magnitude as it appears in sea water(9,10). Trace quantities of Fe and Zn are found normally in the soft tissues of clams; Fe is found in the hemoglobin of corpuscles of many species; and both Fe and Zn are found in kidney and liver (9,11). Thus, uptake of these radioactive elements is probably on an exchange basis with the same elements, or involves replacement of a chemically similar element already in the organism.

Rate of uptake of Co^{60} by the clam's soft tissue and shell was found to be a function of the level of radioactivity of the medium (Fig. 3). A plot of the log rate of uptake ($\mu\text{g/g}$ tissue or shell/day) as a function of the log of concentration ($\mu\text{g/cc}$) in the solution indicates that the relationship of the 2 variables may be described by a power function. This finding agrees with the results reported by Rosenthal(12) on rate of uptake of Ca^{45} by guppies as a function of isotopic concentration and of the uptake of Sr,

† Incorporation of radionuclides in individual tissues was shown by autoradiographs.

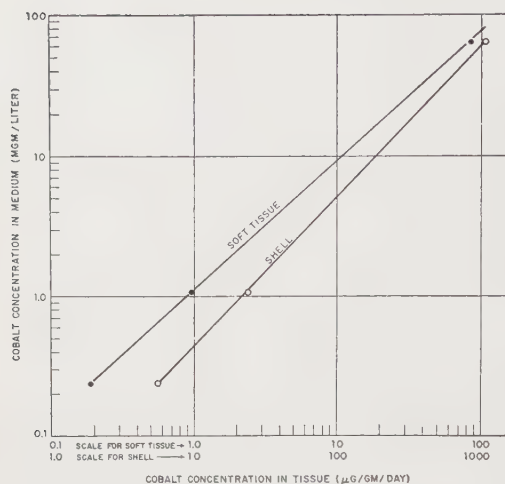


FIG. 3. Rate of uptake of cobalt by the clam vs conc. of the cobalt in the medium (each point represents four clams).

Ba, and La in the goldfish(13).

Summary. A study of the uptake of 3 neutron-induced radionuclides (Zn^{65} , Fe^{59} , and Co^{60}) and a 2-month-old mixture of fission products by the clam demonstrated the ability of this marine animal rapidly to concentrate significant quantities of these radionuclides in both the shell and soft tissue. The uptake of radioactive material in the soft tissue is a metabolic incorporation, while the higher uptake by the shell appears to be a surface adsorption phenomenon. Rate of incorporation by the soft tissues was constant and was highest for the rare earth group of fission products. Uptake of Co^{60} , Zn^{65} , and Fe^{59} was also constant but occurred at a much lower rate. Sr^{89} and $Ru^{103-106}$ were taken up only in equilibrium with the radioactive medium in the experimental interval studied. Further study with the radionuclide Co^{60} indicated that rate of uptake varied as a power

function with level of concentration of the radionuclide in the medium.

The ability of the clam to concentrate radioelements to a high degree makes it valuable as a biological indicator of radioactivity. It may be particularly useful in assessing contamination of marine areas with low levels of radioactivity.

The authors wish to express their appreciation for the technical assistance of R. R. Odum, HN, USN.

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Cultivation of Adult Teleost Tissues *in vitro*. (23250)

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Cultures of normal and abnormal tissue cells from warm-blooded vertebrates are routinely prepared in many laboratories, and there is abundant literature on the subject. In contrast, there is comparatively little information on culture or tissues from cold-blooded vertebrates. Osowski(1) observed cellular movement in fragments of tadpoles and trout which he maintained for as long as 24 hours in Ringer's solution. Sea water and fish bouillon were used as culture medium in other work with fish tissue, but the cultures were short-lived(2,3). Chlopin(4) cultured tissues from pike and crucian carp in rabbit plasma "diluted with homogeneous extract." Lewis and MacNeal(5) cultured pituitary glands from flounder, sculpin and angler fish. Their cultures were "prepared in autoplasm or homoplasma" to which chicken plasma was added. Recently, abnormal tissues of killifish (6) and goldfish(7) were investigated, and in these studies embryonic extracts were added to media composed of chicken, human cord or fish sera and salt solutions. Soret and Sanders(8) propagated the virus of eastern equine encephalomyelitis in fish embryos. There is no information on the culture of tissue cells from salmonids which require low environmental temperatures (10-15°C is the optimum range). Because the culture of host tissues would seem to offer promise as an effective tool in the study of those diseases of salmonids, suspected of being caused by a virus, the initial problem in this investigation was to develop simple and efficient methods of culturing tissues from normal fish. This communication describes the attempts that were made to cultivate salmonid and other fish tissues in certain commercial media.

Materials and methods. Tissues from hatchery-raised rainbow trout (*Salmo gairdneri*) were used for most of the work, but tissues of eastern brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and goldfish (*Carassius auratus*) were also cultured. Age of donors ranged from embryonic

to adult, but cultivation of tissues from sub-adult and sexually-mature specimens was emphasized, because embryonic salmonid tissue is usually available only part of the year. Fish were killed just before use by severing the vertebral column immediately back of the head. Care was taken not to penetrate the body cavity. When gill tissue was needed, the arches were excised and washed in a stream of sterile salt solution from a syringe, which probably helped to remove surface bacteria. When sterile internal organ tissue was needed, the fish was immersed for 1 min. in a 1:1000 solution of benzalkonium chloride (Roccal). After draining, excess disinfectant was removed with cotton swabs saturated with 70% ethanol. Instruments were sterilized in boiling water followed by immersion in 70% ethanol and flaming immediately before use. Tissues or organs were removed aseptically, immediately submerged in cold Earle's solution, and maintained at about 5°C until minced with scalpels and placed in culture vessels. Mincing and all subsequent operations were performed in a hood. Throughout the manipulations the tissues and vessels were kept cool on or near trays of ice, partly because growth of trout tissue was not obtained at 26°C and above and partly because failure in early attempts to obtain growth was attributed to high temperatures within the hood during explantation. Because ozone and ultraviolet light also seemed to be deleterious, the hood was thoroughly flushed with fresh sterile air before use. Some tissues were cultivated directly on glass, but better results were obtained when tissues were embedded in a chicken plasma clot. Cultures were made in straight-necked 5 cm Carrel flasks or stationary 16 x 150 mm tubes, either with or without coverslips. Several 2-3 mm² x 1 mm explants were used in each culture vessel, with 1.5-2 ml of fluid media in flasks, 1-1.5 ml in tubes. When cultures were to be kept a week or longer, medium was changed in whole or in part every 4-7 days. Viability

could often be maintained with weekly renewals. Media used in this work always contained embryo extracts, generally at a level of about 5%. The media also contained from 10-20% serum, and the balance was medium 199 and salt solution with phenol red at 0.001%. Media for internal tissues contained 250-1000 units potassium penicillin G/ml, but penicillin alone did not inhibit contaminants in cultures of gill or fin tissue. Contamination in cultures of external tissues was controlled with 1000 units of penicillin plus 1000 mcg of dihydrostreptomycin sulphate/ml. Best results were usually obtained when the initial pH was adjusted with 5% CO₂ in air to 7.2-7.4, but growth was also obtained at pH 6.8-7.0. Temperature of incubation was 19°C. This investigation did not involve serial transplantation, but some cultures of trout tissue were kept growing for a month and longer, and one culture of goldfish tissue was kept alive for about 10 months.

Results. In general, trout tissues were readily cultivated, but comparable goldfish tissues grew more vigorously. It was necessary to keep trout tissues cool during removal, manipulation and incubation. Swim bladder from eastern brook trout grew well at 12° and 19°C, but more abundant growth was obtained at the latter temperature. No growth of trout tissue occurred at 26° and 37°C. (Maximum temperature tolerance for trout is generally below 26°C.)

Synthetic medium 199 (SM 199) (Difco) and salt solutions were compared for their ability to maintain normal morphology in rainbow trout blood cells. Ringer's frog saline and Holtfreter's solution(9) proved to be unsatisfactory and appeared to be hypotonic. Earle's, Tyrode's, and Hanks' salt solutions and SM 199 gave satisfactory results. Earle's salt solution and SM 199 were judged to be best, and a mixture of the two was used in media for most subsequent fish tissue culture. Growth of trout tissue was obtained in media containing serum, embryo extract and either SM 199, Tyrode's or Earle's, but the results with Tyrode's were inferior. Growth was obtained with chick embryo extract (Difco, desiccated), but a saline extract

of newly-hatched trout gave similar results in cultures of trout kidney and minced trout embryos. Beef embryo extract (Difco, desiccated) also supported growth of fish tissues. Either chicken serum or heparinized trout plasma was employed in much of the work, and serum from carp (*Cyprinus carpio*) supported trout kidney tissue. Many sera supported growth of trout swim bladder tissue, but results with horse serum were poor. Sheep and beef sera were slightly better. Chicken and rabbit sera gave fair results, and human serum was as good as homologous serum. Cellular quality and quantity were excellent in human cord serum; bovine amniotic fluid gave healthier cells, but multiplication was slower than in cord serum.

Tissues from kidney, spleen, peritoneum, gills, testes, ovary, fins, and swim bladder of trout were readily cultured. Heart and liver tissues of trout were less easily cultured. Tissue from a common idiopathic granuloma of eastern brook trout(10) yielded a few abnormal fibroblasts. Heart and kidney of goldfish were easily cultured; one heart culture was kept beating for 299 days in 20% chicken serum, 5% embryo extract, 30% SM 199, and 45% Earle's solution. It died when trout serum was substituted for chicken serum in the medium. Swim bladder explants usually exhibited smooth muscle movement for at least several days; one culture, for over 7 weeks. Ciliary movement was usually observed in explants of swim bladder, kidney and spleen. It was often maintained for several weeks, and for 58 days in one culture of trout swim bladder. Other workers(5) have made similar observations of cilia in fish tissues. Occasional individual cells and aggregations of cells which bore cilia separated from the explant and became motile. This phenomenon has been observed in frog epithelium by Duryee and Doherty(11). With the exception of fin epithelium, cells from tissues in a plasma clot usually adhered better and maintained a healthy appearance longer than those from the same tissue cultured directly on glass. Actively proliferating cultures usually produced only a slight drop in the pH of the medium.

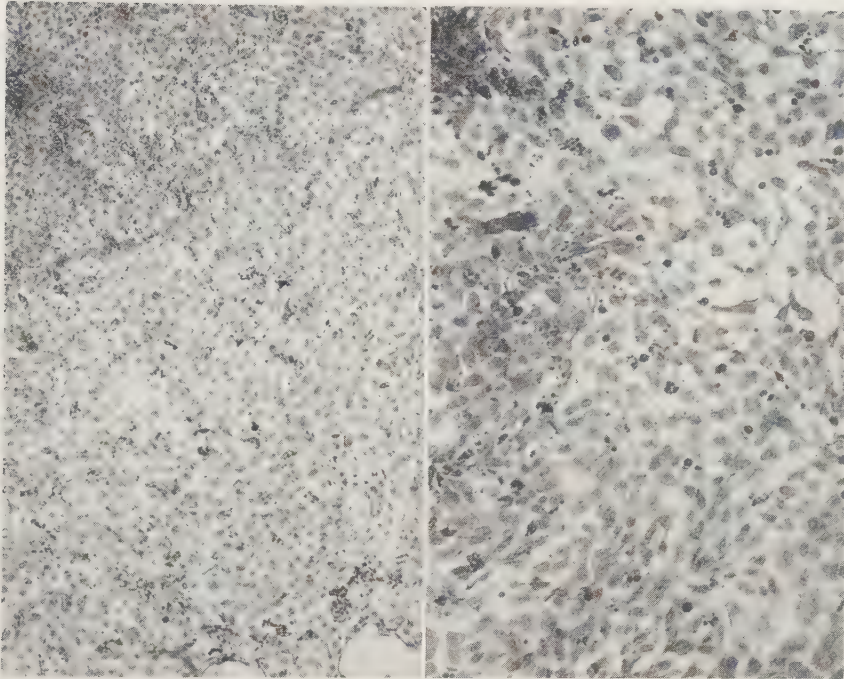


FIG. 1 (left). Cell sheet from 48 hr cultivation on glass of rainbow trout fin. H. & E. $\times 57$.

FIG. 2 (right). Cells from rainbow trout fin in clot after 48 hr incubation. H. & E. $\times 140$.

Gill, swim bladder, kidney and fin tissues from trout regularly gave good cultures. Migration of macrophages was usually evident within hours after explantation and often became extensive. Migration was usually followed by a sheet-like growth of epithelium-like cells (Fig. 1). These sheets grew between the clot and the glass, and at times over the surface of the clot. Sheet-like growth of epithelium was often the first activity of fin, gill and swim bladder tissues. Cells comprising these sheets were polygonal to smoothly contoured in outline (Fig. 2). Their early appearance was hyaline, but with age and especially with media containing chicken serum they became granular and vacuolated. Sheets of older cells often tore or developed holes, which were rapidly covered by a sheet of new cells, and several such cycles were observed in a single culture. Hyaline spindle-shaped cells or cells with multiple processes were the last to appear. This cell type usually overwhelmed earlier appearing cells.

Summary. Normal tissues from trout—a

coldwater teleost—have been successfully cultured for as long as 65 days at 19°C or lower in 20% serum, 30% SM 199, 45% Earle's solution and 5% embryo extract. Excellent results were obtained with human cord, human, homologous sera or bovine amniotic fluid when used at 20% level by volume. Manipulation and incubation temperatures below 22°C are thought to be essential.

Appreciation is expressed to Dr. S. F. Snieszko, U. S. Fish and Wildlife Service, for his kind interest, and to Dr. Karl Habel, National Institute of Allergy and Infectious Diseases, for instruction in methods of tissue culture.

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A Simple Method for Determination of Desoxypentose Nucleic Acid in Tissue Cultures. (23251)

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The numerous methods now employed for quantitative determination of desoxypentose nucleic acid (DNA) suffer from either a laborious purification procedure or the capriciousness of colorimetric assays for desoxypentose(1,2,5-8,10-14,16). The desirability of quantitation by means of ultraviolet absorption has been recognized(8,10,14), but these methods are either too laborious or their specificity has been questioned(7).

A new method is presented here which consists of a very simple, *but highly specific*, purification of DNA and quantitation by means of the ultraviolet absorption. The basis of the method is the fact that DNA, in concentrations as low as 5 μ g per ml, can be quantitatively precipitated from .04 N sodium hydroxide solution by salmine. Interference by ribonucleic acid is completely eliminated by alkaline hydrolysis(12,15) before the DNA precipitation. The purines and pyrimidines are recovered from the salmine precipitate by extraction with an acid salt solution. DNA recovery is nearly 100%. The method as presented is designed for a lower limit of 2 μ g of DNA. By manipulation with smaller volumes a lower limit of 0.2 to 0.5 μ g should be feasible.

Experimental. Reagents are 0.4 N sodium hydroxide; 0.1 N sulfuric acid containing 10% sodium chloride (C.P. grade); 1% solution of salmine (General Biochemicals Inc.) **Procedure.** 1. Separate cells from culture medium and wash generously with physiological salt solution. For cultures firmly adhering to glass, centrifugation is not necessary and two 10 ml washings are more than adequate for

cultures of 1 to 5 x 10⁶ cells. 2. Take up the cells in a small volume of 0.4 N sodium hydroxide; heat in boiling water 1 hour, with a glass cap over the neck of the culture tube to minimize evaporation; cool and dilute the sodium hydroxide to 0.04 N. The volume of 0.4 N sodium hydroxide required in the cell extraction depends upon the number of cells in the culture. If too much alkali is used, dilution to 0.04 N will give a solution too dilute for quantitative precipitation of the DNA. If too little alkali is used RNA will not be completely hydrolyzed and a high value for DNA will be obtained. The best solution to this problem is to have an extra replicate culture for "range-finding." Extract the extra replicate with 5 ml of the acid salt solution in boiling water for 30 minutes, cool, centrifuge and read the absorbance at 268 and 330 m μ . Use the following guide for the alkali extraction:

Absorbance, 268 m μ -330 m μ	ml of 0.4 N NaOH	Dilution after heating
.050 to .100	.05	.5 ml
.100 .200	.1	1
.200 .400	.2	2
.400 .800	.4	4

Cultures of 2 to 3 x 10⁶ strain L cells will require 0.4 ml of 0.4 N sodium hydroxide for the extraction. 3. To 0.4 ml of diluted cell extract, in a 0.4 ml graduated test tube, add 0.02 ml of salmine solution.* Mix thoroughly

* Volumes less than 1 ml were measured with micropipets made by Microchemical Specialties Co. The 0.4 ml test tubes were 8 mm O.D., 70 mm long, graduated by Paul O. Moeller, Universal Instrument Corp., Chicago.

but gently (avoid foaming) and chill on ice for 1½ hours. Centrifuge for 10 minutes at 2000 rpm and 0 to 4°C; draw off the supernatant and wash carefully with 0.4 ml of ice cold water. Drain the tube in an inverted position for a few minutes and then shake out the last drop of water. 4. To the tube containing the precipitate add slightly less than 0.4 ml of acid salt solution, cover with a glass cap, heat in boiling water 30 minutes, cool, centrifuge briefly, dilute carefully to 0.4 ml with distilled water, mix well and centrifuge to pack down the slight precipitate. 5. Measure absorbance of the acid saline extract at 268 and 330 mμ. (A Beckman, Model DU spectrophotometer, equipped with a Lowry and Bessey micro cell(9), is recommended.) The reading at 330 mμ is a measure of a very slight haze which may be present. In most cases the 330 mμ reading is so small that it could be ignored. The difference between the 2 readings is proportional to amount of DNA in the original sample (Fig. 3). The quantity of DNA per culture may be expressed in any one of several convenient ways. As a standard of reference we used a preparation of calf thymus DNA purified(3,4) to an analysis of 14.9% nitrogen and 8.53% phosphorus. This DNA preparation, at a concentration of 10 μg per ml, dissolved by heating in the acid salt solution, had an absorbance of 0.260 at 268 mμ (1 cm light path). The amount of DNA per culture may be calculated in terms of the reference standard as follows: A = Absorbance reading, V = Total vol. of 0.04 N NaOH extract.

$$\mu\text{g DNA/culture} = V \times \frac{A_{268 \text{ m}\mu} - A_{330 \text{ m}\mu}}{0.026}$$

Specificity of method. Fig. 1 shows the absorption spectra of the acid saline extracts of the salmine precipitates of purified DNA, HeLa extract and strain L extracts. (HeLa cells were obtained through the courtesy of Dr. William F. Scherer, Univ. of Minnesota; strain L, NCTC929-209-7, was obtained through the courtesy of Dr. Wilton R. Earle, Nat. Cancer Inst.) The spectrum of a 0.5% solution of salmine is included to show that this protein contributes nothing to the absorption of the extracts above 250 mμ. A high

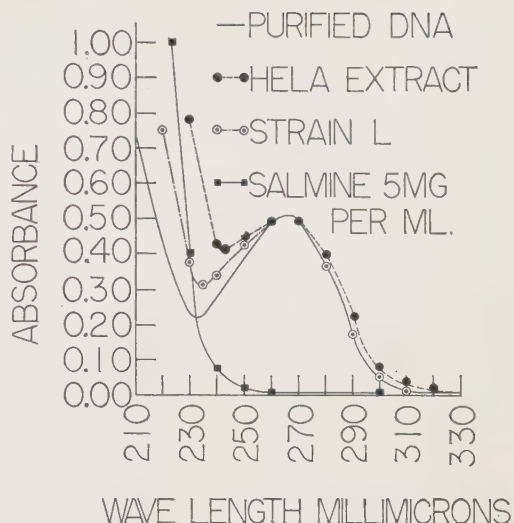


FIG. 1. Ultraviolet absorption spectra of acid saline extracts of salmine precipitates ("DNA extracts") from purified DNA and from alkaline extracts of HeLa and strain L cells.

degree of specificity for the method is indicated by the fact that the HeLa and strain L curves follow very closely the curve for purified DNA from 250 mμ on up to 330 mμ. As a further test of specificity the absorption spectra of these same extracts were obtained under alkaline conditions (~0.1 N sodium hydroxide). The tyrosine absorption peak of proteins, which appears at 275 and 280 mμ under acid conditions, is shifted to 290 mμ and intensified under alkaline conditions. If there were enough protein in the "DNA" extracts to contribute significantly to the absorption at 268 mμ under acid conditions, this protein interference should show up as a hump at 290 mμ in the absorption curve under alkaline conditions. Also, the absorption at 290 mμ should be greater in alkali than in acid. That such is not the case is shown in Fig. 2. Both HeLa and strain L "DNA" extracts show smooth absorption curves above 270 mμ in alkali, and both alkaline curves are slightly less intense than the corresponding acid curves. Below 250 mμ there is a much greater difference between acid and alkaline absorption curves of conalbumin. In this range also there are relatively slight differences between the acid and alkaline absorption curves of HeLa and L "DNA" extracts. It is therefore clear that protein

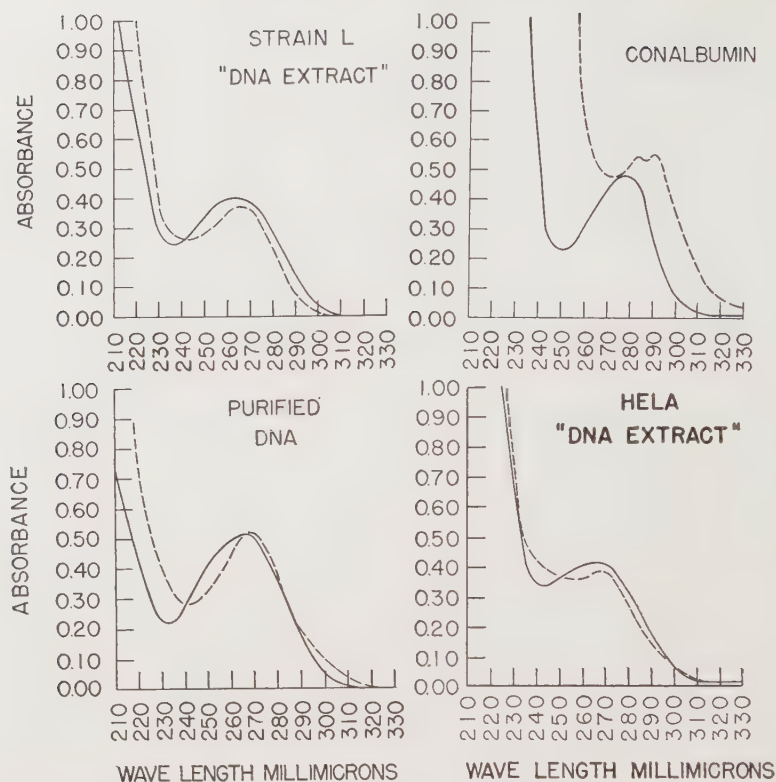


FIG. 2. Comparison of absorption spectra in acid (0.1 N H_2SO_4), solid lines, to those in alkaline solution (0.1 to 0.2 N $NaOH$), broken lines.

interference in this DNA determination is negligible. Conalbumin was chosen as a representative protein in these studies because it contains only moderate amounts of tyrosine and tryptophane. Insulin, which contains more tyrosine than conalbumin shows much greater differences in absorption between acid and alkaline solutions. Further evidence for the specificity of the salmine precipitation for DNA was obtained from the sugar color reactions. The orcinol test indicated that salmine precipitates from HeLa and strain L extracts contained negligible pentose. The diphenylamine reaction indicated that the salmine precipitates of HeLa and strain L extracts contained as much desoxypentose as the salmine precipitate of purified DNA, in relation to purine and pyrimidine content. The recovery data shown in Table I attest to the specificity of the salmine precipitation of DNA. There was no coprecipitation of either the mono nucleotide, cy-

tidylic acid, or the alkaline hydrolytic fragments of RNA.

Reproducibility of the method and recovery of DNA. That the reproducibility of the method is very good is indicated by the data of Table I. The recovery of DNA is excellent alone or in the presence of alkali hydrolyzed RNA, alkaline cell extracts, or cytidylic acid. Of the 38 recovery figures reported, the mean value is $97.85 \pm 0.87\%$ (standard error). Fig. 3 shows the relationship at 268 mμ between absorbance of the acid saline extract of the salmine precipitate, and amount of DNA before precipitation. Each point on the graph represents the average of the 4 absorbance readings from which the corresponding recovery figures in Table I were calculated.

Summary. A new method for quantitative determination of desoxypentose nucleic acid has been presented. The innovation of this method is precipitation of DNA by salmine

TABLE I. Recovery of DNA in Presence of Thymidylate, Alkali Hydrolyzed RNA and Alkaline Cell Extracts.*

Constituents of 0.04 N NaOH solution	DNA added (μg)	DNA found (μg)†				
NaOH alone	5.44	5.16 (94)	5.44 (100)			
5.76 μg RNA‡	5.44	5.4 (99)	5.12 (93)			
20 μg calcium thymidylate	5.44	5.4 (99)	5.24 (96)			
40 μg " "	none	.31	.32			
11.5 μg RNA	"	.1	.1			
	21.8	21.6 (99)	21.8 (100)	21.6 (99)	21.8 (100)	
	10.9	10.8 (99)	10.8 (99)	10.8 (99)	10.7 (98)	
	4.36	4.1 (94)	4.1 (94)	4.18 (96)	4.2 (96)	
	2.18	1.98 (91)	1.98 (91)	2.16 (99)	2.11 (97)	
Strain L extract	none	3.48	3.58	3.6	3.66	
	10.9 §	14.93 (104)	15.31 (108)	14.78 (103)	14.48 (100)	
	2.18§	5.88 (106)	5.85 (104)	5.76 (100)	5.59 (92)	
HeLa extract		3.84	4.0	3.97	3.97	
	10.9 §	14.24 (94)	14.94 (101)	14.04 (93)	13.54 (88)	
	2.18§	6.07 (98)	6.41 (113)	5.83 (87)	6.16 (102)	

* Each "DNA found" value represents an individual determination, not an avg value.

† Figures in parentheses give % recovery.

‡ Hydrolyzed in 0.4 N NaOH, 60 min., 100°, then diluted to 0.04 N NaOH.

§ For calculation of DNA recovery from HeLa and strain L extracts, avg values of 3.58 μg DNA for strain L and 3.94 μg DNA for HeLa were used.

and dissolution of purines and pyrimidines from the precipitate by means of hot acid salt solution. The quantitation depends upon the absorption of the acid saline extract at 268 μm . The method is very specific for DNA. Precision and DNA recovery are both

good.

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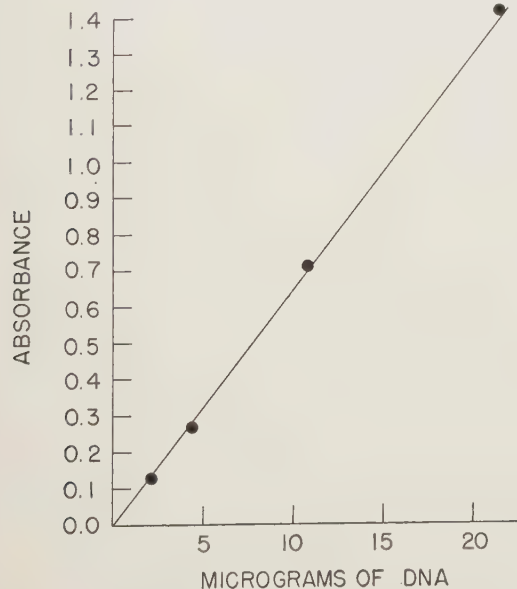


FIG. 3. Absorbance of acid saline extract of saline precipitate as related to amt of DNA before precipitation.

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Relationship Between Sleep, Biotransformation Rates, and Plasma Levels of Pentobarbital and Secobarbital in Animals. (23252)

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Pentobarbital (Nembutal) (5-ethyl-5-(1-methylbutyl)-barbiturate sodium) and secobarbital (Seconal) (5-allyl-5-(1-methylbutyl)-barbiturate sodium) are very similar in structure and both are classed as "short-acting" in duration of action(1). Pentobarbital has been widely studied in animals with regard to its fate and metabolism, but there is little comparable data on secobarbital. Man seems to be the only species studied(2,3) in this manner.

It was our purpose to compare these 2 barbiturates with regard to sleeping time, body or plasma levels at the time of waking and rate of destruction in mice, rabbits, and dogs given single doses (equivalent by weight) of pentobarbital and secobarbital.

Materials and methods. The molecular weights of pentobarbital and secobarbital are within 5% of each other. This slight difference would not be evident in experiments that have biological errors which are considerably larger than 5%, so equivalent doses in weight per kilo were used throughout this work. Groups of female, albino mice, weighing between 18-22 g were injected intraperitoneally with either barbiturate as the sodium salt made to a 2% solution. Sleep was recorded as the interval between the time mice remained on their back to the time they woke and rolled over. For experiments measuring both body level and sleeping time of drug, each mouse was killed by impact, on waking, the whole animal homogenized in a Waring blender and an aliquot analyzed for barbiturate. To determine rates of destruction, separate experiments were run with groups of mice analyzed at definite time intervals after

injection regardless of whether they were asleep or awake. A group of female Sprague-Dawley rats weighing between 230-330 g was injected intraperitoneally with each barbiturate and sleeping time (righting time) recorded. One week later those animals that had received pentobarbital were given secobarbital and those that had received secobarbital were given pentobarbital. This cross-over arrangement equalized the effect of time and tolerance and the relatively large animal to animal variation was minimized. This design was used in some other experiments also (see below). Only sleeping times were studied on rats. Male rabbits weighing between 2-4.8 kg were injected intravenously with a 5% solution of the sodium salt of each barbiturate. Sleeping times were recorded from the time of injection to the time they righted themselves from a side position. Blood samples were taken by heart puncture at 1 and 2 hours and at the time of awaking. They were centrifuged, and an aliquot of the plasma analyzed for the barbiturate. Preliminary experiments showed that the plasma and tissues had reached equilibrium before one hour. One experiment was set up on a cross-over design as described above with a 5-day interval between experiments. Female mongrel dogs weighing between 5 and 12 kg were injected intravenously with 3% solutions. Blood samples were drawn from leg veins at 1 and 4 hours and at waking time and the plasma analyzed. The experiment was set up in a cross-over design as described above for rats with one week intervals between experiments. Pentobarbital and secobarbital were determined by the method of

TABLE I. Comparison of Action of Pentobarbital and Secobarbital in Mice Given Varying Doses Intraperitoneally.

Dose, mg/kg	No. of mice	Pentobarbital				Secobarbital			
		Sleeping time, min.	Whole mouse content at waking, $\mu\text{g/g}$	% dose recovered	(Calculated) destruction, %/hr	No. of mice	Sleeping time, min.	Whole mouse content at waking, $\mu\text{g/g}$	(Calculated) destruction, %/hr
40	10	24 \pm 6*	33 \pm 4	66 \pm 8	137	10	24 \pm 9	24 \pm 4	93
50	9	20 \pm 6				10	59 \pm 30		
60	10	74 \pm 17				10	90 \pm 18		
80	10	93 \pm 21	29 \pm 7	36 \pm 9	69	9	168 \pm 23	19 \pm 4	53
80	30				78†	30			55†
100	10	124 \pm 24	28 \pm 4	28 \pm 4	64	10	275 \pm 82	41 \pm 4	28
120	8	179 \pm 41	27 \pm 5	23 \pm 4	51	10†			

* Mean \pm stand. dev.

† Data from Fig. 1.

‡ Nine out of 10 died.

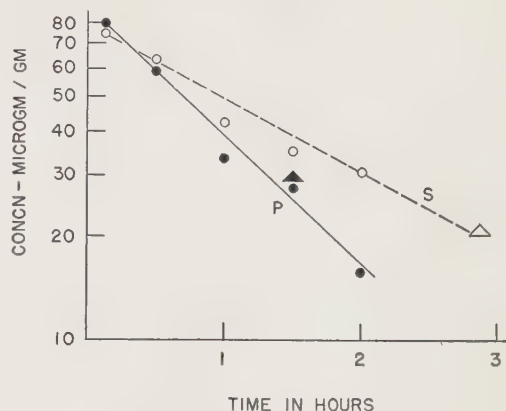


FIG. 1. Whole animal levels of pentobarbital (●—●) and secobarbital (○—○) in mice inj. intraper. with 80 mg/kg. Each point is the avg of 10 analyses on 10 mice. Avg time of waking and whole animal level of pentobarbital (▲) and secobarbital (△).

Brodie *et al.*(4). Recoveries of either barbiturate added to rabbit plasma or whole mice ranged between 95-97 and 93-99%, respectively. Preliminary experiments not reported here established that in the rabbit and dog, the plasma barbiturate levels decreased in the usual logarithmic straight line manner between the time intervals reported here. The rates of decrease in terms of per cent per hour were calculated from these 2 points, averaged, and the standard deviations calculated on the assumption that the rates in terms of per cent per hour were normally distributed. Plasma levels at waking time were averaged on the assumption that they were normally distributed.

Results. The data on rate of destruction of a single dose of 80 mg/kg pentobarbital and secobarbital injected intraperitoneally into mice are summarized in Fig. 1. Mice receiving pentobarbital slept a significantly shorter time (93 \pm 21 (S.D.) min. *vs.* 168 \pm 23 min.) ($P = < .001$) than those receiving secobarbital. Pentobarbital was destroyed at a significantly greater rate than secobarbital (78 \pm 15 *vs.* 55 \pm 7 (S.D.) %/hr respectively) ($P = < .001$). In another experiment it was shown that at waking time, mice receiving pentobarbital exhibited higher whole animal levels than those receiving secobarbital (29 \pm 7 (S.D.) *vs.* 19 \pm 4 $\mu\text{g/g}$ respectively) ($P = < .01$). The brains of mice re-

TABLE II. Comparison of Pentobarbital and Secobarbital in the Rat, Rabbit and the Dog.

Species	Dose, mg/kg	No. of animals	Pentobarbital			Secobarbital		
			Sleeping time, min.	Plasma conc. at waking, $\mu\text{g/ml}$	Rate of destruction, %/hr	No. of animals	Sleeping time, min.	Plasma conc. at waking, $\mu\text{g/ml}$
Rat	20	20*	89 \pm 28†			20*	77 \pm 29	
	30	19*	207 \pm 27			19*	204 \pm 29	
Rabbit	15	10	41 \pm 12	9.6 \pm 1.1		5	36 \pm 4	9.5 \pm 1.0
	25	15*	89 \pm 19	9.6 \pm 1.7		15*	104 \pm 21	7.9 \pm 2.0
	25	5	114 \pm 36	10.5 \pm 2.0	35 \pm 10	5	133 \pm 44	9.0 \pm 1.3
Dog	20	6*	52 \pm 19	14.8 \pm 1.6		6*	89 \pm 32	11.2 \pm 1.4
	25	6*	104 \pm 38	17.2 \pm 2.2	15 \pm 2	6*	183 \pm 46	12.3 \pm 1.1

* Cross-over experiment (see text for explanation). Rabbits—5 day interval between experiments. Dogs—7 day interval between experiments.
† Mean \pm stand. dev.

ceiving pentobarbital were also analyzed at waking. The brain pentobarbital/g wet wt/body (minus head) (pentobarbital/g wet wt) ratio of 10 mice averaged 1.01 ± 0.34 (S.D.).

Table I summarizes the data on sleeping time, calculated rate of destruction and whole animal levels of pentobarbital and secobarbital at waking time in mice given different doses of each. At the lower doses of 40 and 60 mg/kg the longer sleeping times of mice given secobarbital were not statistically significant ($P = >.05$) but at doses of 50, 80, 100 and 120 mg/kg mice slept significantly longer ($P = <.01$). Whole animal levels at awaking were relatively constant for pentobarbital regardless of dose or length of sleeping time, but secobarbital whole animal level values at waking showed a greater variation. At doses of 50 and 80 mg/kg the whole animal level at waking was significantly higher with pentobarbital than with secobarbital ($P = <.01$). However, at 100 mg/kg the situation was reversed. This high secobarbital value may be caused by the relatively longer sleeping time of $4\frac{1}{2}$ hours on this high dose.

Assuming that the decline in whole animal content is logarithmic with time (as shown in Fig. 1) and that at injection 100% of the drug is in the animal one can calculate rate of destruction. On the basis of per cent per hour destroyed it is evident that rate of destruction tends to be dependent on the dose. As the dose increased the rate decreased for both barbiturates. This is unusual because similar studies of barbiturates in other species indicate that rate of destruction is independent of dose. However, at all doses studied, pentobarbital was destroyed at a more rapid rate than secobarbital.

Table II summarizes similar data on rats, rabbits, and dogs. There was no significant difference in sleeping time between these 2 barbiturates at either 20 or 30 mg/kg in rats receiving the drug intraperitoneally.

At a dose of 15 mg/kg in rabbits (I.V.) there was no significant difference in either sleeping time or plasma level at waking between these 2 drugs. However, when a larger dose of 25 mg/kg was used, rabbits slept a

shorter length of time on pentobarbital (89 ± 19 (S.D.) *vs.* 104 ± 21 min.) ($P = <.01$). These animals woke with slightly higher pentobarbital plasma levels than those receiving secobarbital (9.6 ± 1.7 (S.D.) *vs.* 7.9 ± 2.0 mg/g) ($P = <.01$). In a second experiment of 5 animals it was found that rate of destruction of pentobarbital and sleeping time again were less than that of secobarbital. Because of the small number of animals the difference in sleeping time was not statistically significant. At the dose of 15 mg/kg the brain/plasma ratio in 15 rabbits at waking time was 1.66 ± 0.2 (S.D.) for pentobarbital and 1.61 ± 0.1 for secobarbital.

At an intravenous dose of 20 mg/kg dogs slept a shorter time and woke with higher plasma levels with pentobarbital than with secobarbital, but the differences were not statistically significant ($P = >.05$). However, at a higher dose of 25 mg/kg the shorter sleeping time and the higher plasma levels with pentobarbital became significant ($P = <.01$). Rate of destruction of both of these compounds is almost identical (15 ± 2 *vs.* $14 \pm 3\%$ per hour).

Discussion. Our values for pentobarbital plasma levels at waking are essentially in agreement with those found by Goldbaum and Schack(5). At a dose of 30 mg/kg intravenously in their experiments, rabbits woke with plasma levels of 12 ± 0.4 (S.D.) $\mu\text{g/ml}$. Our results substantiate the results of these investigators in that there was no significant correlation between duration of sleeping time and plasma levels at waking in rabbits. However, there is a species difference for pentobarbital plasma level at waking. A survey of the recent literature(6) together with results of this investigation indicates that the critical plasma level at waking decreased in the following order: mouse $>$ rat $>$ dog $>$ rabbit $>$ man.

This work emphasizes the importance of comparing 2 barbiturates at more than one dose level in the mouse, dog and rabbit in order to determine potency by sleeping time response. At low doses no statistically significant differences could be demonstrated in these animals, but at higher doses a statis-

tically significant difference became apparent. In addition, the slopes of the response curves differed in these 2 barbiturates. Thus, in addition to specifying the species studied, one must also specify the dose level studied with these 2 barbiturates.

A significant and unusual observation noted in this work was that rate of destruction of both barbiturates in mice decreased with increasing dose. Ordinarily rate of destruction of a drug is considered to simulate a first order reaction in which amount of drug destroyed, is proportional to amount of unchanged drug present, and is independent of dose. A survey of the literature indicates that the problem of comparison of rates of barbiturate destruction at different doses has not been investigated to any extent. We hope to investigate this phenomenon further because of the widespread use of the mouse in problems of barbiturate sleep potentiation.

Summary. Pentobarbital and secobarbital were compared in equivalent doses in the mouse, rat, rabbit and dog. When given pentobarbital, female mice destroyed it faster, slept a shorter length of time, and woke at higher body levels than with secobarbital. Female rats showed no significant difference in sleeping times between these 2 drugs. Male rabbits and female dogs slept a shorter length of time and woke with higher plasma levels with pentobarbital than with secobarbital. There was no statistically significant difference in rate of destruction between these 2 drugs in these 2 species. In the mouse rate of destruction of both drugs was dependent on size of the dose, the higher the dose, the lower the rate of destruction. Pentobarbital and secobarbital had different dose-sleeping time response curves in mice, rabbits and dogs (but not rats).

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Desoxyribosenucleic Acid as Index of Mammary Gland Growth of Mice.*† (23253)

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Several methods have been proposed to estimate quantitatively mammary gland growth (1,2). Since the suggestion(3) that desoxyribosenucleic acid (DNA) may be constant in the somatic cells of various tissues of any species, DNA has been employed as a measurement of somatic growth and as an index of mammary gland growth in rats(4) and rabbits(5). Recently Griffith and Turner (6) have shown that DNA is constant per nucleus in mammary gland and in other somatic tissue cells of rats and mice during pregnancy. Further, it has been shown that changes in collagen content of the mammary gland are small in comparison to changes in DNA, indicating that most of DNA change during growth is due to mammary parenchymal increments(7). With the establishment of DNA as an accurate measure of mammary gland growth and a sensitive technic of determination available(8), the use of male mice was explored as assay animals for the determination of mammary gland stimulating hormones.

Methods. Male albino mice weighing 16-18 g at start of the experimental periods were used. In first experiment, groups of mice were fed a ration containing 1.23 mg diethylstilbestrol/kg ground mouse food for periods of 0, 1, 2, 3 and 4 weeks, and sacrificed after each period. In second experiment, mice were maintained on a similar diet for 4 weeks in order to insure good development of the mammary duct system(9), then divided into

groups receiving graded amounts of estradiol benzoate plus progesterone subcutaneously for 10 days, using a 1:1000 ratio of the 2 substances. This particular ratio has been reported to be optimal in the production of mammary lobule-alveolar development in mice(10,11). Estradiol benzoate (0.75 µg/day) alone was injected into one group for the same length of time. Mice were sacrificed approximately 24 hours following the last injection, skinned, and $\frac{3}{4}$ of subcutaneous fascia, including mammary glands, were removed for DNA determination. The tissues were quickly frozen by placing the tissue container in an alcohol dry ice bath. The frozen tissues were then lyophilized, fat extracted in hot alcohol and then ether for 12 hours, and ground to a fine powder in a Wiley mill. DNA was extracted from 30 mg dry, fat-free tissue samples by procedure of Schneider(12) with exclusion of the fat extraction step. The tissue was extracted twice with 5 ml of cold 10% trichloroacetic acid (TCA), in a cold room held at 4°C, centrifuged and supernatant discarded. The residue was extracted with 10 ml of hot 5% TCA, and then 5 ml, for periods of 20 minutes. The supernatants were pooled, brought up to a volume of 15 ml, and DNA determined on 2 ml aliquots by the p-nitrophenylhydrazine method of Webb and Levy(8) in preference to the older diphenylamine method of Dische(13). The same purified standard DNA preparation was used throughout all experiments; however, the phosphorous content and the nitrogen content of the preparation were not checked. The remaining quarter of tissue was fixed in Bouin's fluid, washed in water, stained in Delafeld's hematoxylin, differentiated in acid-

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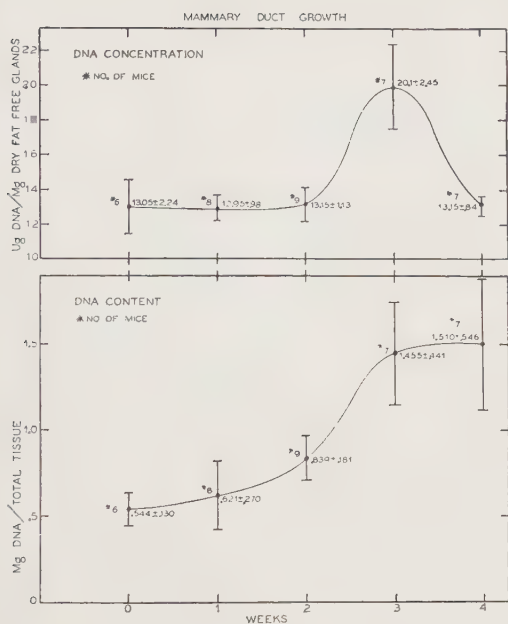


FIG. 1. Relative amounts of DNA in mouse mammary gland during various stages of duct growth. Mice were fed a ration containing 1.23 g diethylstilbestrol/kg. Range represents one stand. error. Note that concentration curve (above) approximates a lagging differential of content curve.

alcohol, and kept in alcohol for visual examination prior to mounting(1).

Results. The DNA of the mammary glands of male mice receiving oral estrogen for varying lengths of time was determined to see if growth could be detected in the developing duct system by this method. Total DNA increased somewhat from the first to the second week, but not until the third week did the DNA rise significantly ($p < .02$) (Fig. 1). There was no difference noted between the total DNA of mammary glands of mice receiving oral estrogen for 3 or 4 weeks. The DNA concentration, expressed as $\mu\text{g}/\text{mg}$ dry, fat-free tissue, remained essentially at the same level until after mice were fed estrogen for 3 weeks. At this time a significant ($p < .001$) increase was observed, after which the concentration decreased to the original level. Whole mount observation indicated progressive, though variable, development of the mammary duct system throughout the feeding period.

The second experiment was devoted to de-

termining the levels of DNA in the mammary gland at various stages of development of the lobule-alveolar system. Total DNA in mammary glands of mice injected with a control amount of estrogen remained at a low level, while in mammary glands of mice receiving graded doses of estrogen plus progesterone, it increased generally as the log-dose of the hormones (Fig. 2). DNA concentration, however, rose rapidly from the level obtained in the estrogen treated group to the level in group receiving $.06 \mu\text{g}$ estradiol benzoate (E.B.) plus 0.06 mg progesterone (P). From this point it rose to the peak amount seen in the group receiving $0.125 \mu\text{g}$ E.B. plus 0.125 mg P and gradually came down in each successively higher dose until it reached the low level seen in the group receiving $0.75 \mu\text{g}$ E.B. plus 0.75 mg P. Whole mount observation again indicated progressive, though variable, development of the mammary lobule-alveolar system with increasing doses of estrogen and

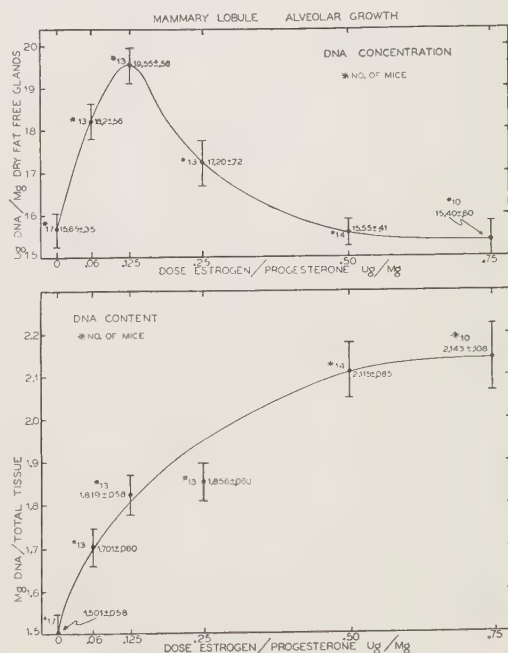


FIG. 2. Relative amounts of DNA in mouse mammary gland at various stages of lobule-alveolar development, stimulated by inj. various levels of estradiol benzoate and progesterone (a constant ratio of 1:1000 was kept) each day for 10 days. Range represents one stand. error. Again concentration curve approximates a differential of content curve.

progesterone.

Discussion. Kirkham and Turner(4) found that DNA content (total DNA) of the mammary glands of rats increased during the first two-thirds of pregnancy, after which time it increased only slightly. This period is generally regarded as the time of greatest mammary gland growth. The results, showing an increase of total DNA during periods of both duct and lobule-alveolar growth, agree well with these findings, in that amounts of total DNA per gland are related to the stage of growth. Further, total DNA accumulation seems to follow a logarithmic progression, in that an approximately straight line is obtained when mean DNA content values are plotted on semilogarithmic paper.

Leslie and others(14) demonstrated that DNA concentration (expressed in terms of DNA/mg protein nitrogen) may be roughly related to rate of growth of tissues, a fact which may be due to the lag in cytoplasmic protein accumulation in rapidly dividing cells. This may be based on the idea that as increasing numbers of cells divide, the DNA increase is much greater than increase in cytoplasmic protein, until the protein "catches up" with the DNA. This seems to agree with results which show that DNA concentration, expressed in terms of μg DNA/mg dry, fat-free tissue, approximates a differential of the DNA content curves.

Several factors may be operative in production of variance in DNA. Among these are the differing responses of the pituitary to progesterone, the differing responses of the mammary gland to pituitary mammogen, and varying secretion rates of other hormones influencing mammary gland growth. Mouse weight was not a factor, in that no significant correlation could be obtained between mouse weight and DNA content of mammary gland. The coefficients of variation compared favor-

ably with previous work(1,5).

Summary. 1. Total DNA of the mouse mammary gland is an index of mammary gland development while concentration (μg DNA/mg dry, fat-free tissue) seems to be an index of growth rate. 2. Feeding 1.23 mg diethylstilbestrol/kg mouse food caused most rapid mouse mammary duct development between the second and third weeks. 3. Rate of mammary gland growth is not constant. At the end of a 10-day period, mice injected with lower doses of lobule-alveolar developing substances seemed to be developing at a faster rate, whereas those receiving a higher dose had a greater amount of absolute growth, although the rate of development had passed the peak.

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Lipid Mobilization by a Crystalline Peptide Isolated from Plasma of Horses Administered Cortisone.*† (23254)

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It has been reported that administration of cortisone resulted in the appearance in the plasma of an agent that inhibits the delactescing action of plasma obtained after heparin administration(1). This observation has been confirmed(2) and a less direct demonstration of this phenomenon has also been reported(3). In addition, plasma and its dialyzate prepared from plasma of horses administered cortisone (LM) caused hyperlipemia when administered to other animals(4,5) and patients(6). This effect occurred in the absence of adrenals or pituitary and differed from other hyperlipemia inducing agents such as protamine, pyrogens and DFP which required the presence of these endocrines(5). In this paper we are reporting data on the potency of crystalline LM.

Materials and methods. The sterile, pyrogen-free dialyzate was prepared as previously described by us(4). Preliminary chemical studies suggested that the active material was a polypeptide. The isolation and purification was in conjunction with our chemistry department and the details will be published elsewhere. Throughout the purification the samples were coded by the chemist and biological assays were run as unknowns. None of the operators analyzed any plasma sample for all lipids. Determinations of potency as a guide for purity were carried out in 865 male and female Wistar strain rats weighing 125-150 g. All animals and patients used in the present

study were permitted only water for the 18-24 hours preceding injection of LM. The methods for determining plasma lipid levels were as previously described(5,6). The dose response curve with the final crystalline material was determined by injecting 10 intact rats at each of 10 logarithmically spaced doses. The minimal effective intravenous (recurrent saphenous vein) dose necessary to produce a 2-fold elevation in plasma cholesterol in 100% of the rats was 0.02 μ g/kg. This dose was then similarly administered to 120 intact rats which were sacrificed in groups of 20 after 1, 2, 4, 6, 12 and 24 hours in order to determine duration of action. In addition, the same dose was injected intravenously into 20 hypophysectomized and 20 adrenalectomized rats, 60 guinea pigs, 6 dogs, 60 intact mice (tail vein), 12 intact rabbits (marginal ear vein) and into 3 patients at Temple University Hospital. Blood samples from the rats, guinea pigs and mice were obtained from the beating heart exposed under light ether anesthesia. All animals and patients were maintained in the fasted state until all blood samples had been obtained. The animals used in this and our previous studies had been subjected inadvertently to one of the following treatments: (1) The dogs were dipped in a chlordan bath; (2) all other animals were periodically sprayed with an insecticide containing piperonyl butoxide; (3) rabbits and guinea pigs received a supplementation of 10 g of chlortetracycline/ton of chow from the time of weaning; (4) similarly, rats and mice received a supplementation of 4 g of procaine penicillin/ton of food which contained in addition 0.005% butylated hydroxyanisole.

Results. Fig. 1 shows dose response relationship between crystalline LM and cholesterol concentration in the plasma. The cholesterol was determined for each rat. It is of interest that rats could respond with an

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TABLE I. Effect of Crystalline LM 0.02 $\mu\text{g}/\text{kg}$ on Plasma Lipids (mg %) of Various Species.

Species	Hr after injection											
	0			1			2			4		
	CH	FA	LP	CH	FA	LP	CH	FA	LP	CH	FA	LP
Rats, intact	64	100	5	188	214	9	302	350	10	302	353	10
" hypex	56	80	5				248	313	11			
" adrex	58	91	5				250	336	10			
Mice	52	90	5	194	238	10	266	408	14	309	364	15
Guinea pigs	85	104	5	185	200	9	250	272	10	335	350	11
Rabbits	64	92	5	196	250	10	248	320	11	332	390	13
Dogs	124	160	6	300	352	11	390	400	18	418	404	19
Humans	220	230	7	390	440	13	420	500	15	490	560	16

CH = Cholesterol. FA = Total fatty acids. LP = Lipid P.

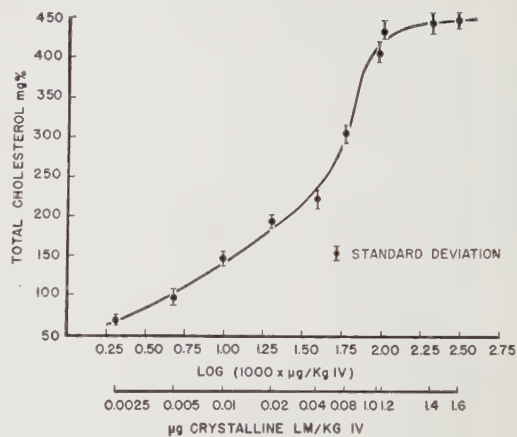


FIG. 1. Dose response of rats to IV crystalline LM (10 rats/dose).

8 to 9 fold elevation of plasma cholesterol shortly after injection with 2000 times the minimal effective dose. Doses larger than this produced no further elevation. Table I presents the values for the plasma concentrations of cholesterol, total fatty acids and lipid P at various times after LM administration into the species tested. The response is manifested after one hour; however, the duration of action of this dose was different for each species.

Discussion. The 10,000 animals used in this and in our previous studies had been exposed to conditions considered to be harmless but which when intensified have in each instance been reported to produce fatty liver (7-11). It would appear, therefore, that the hyperlipemic action of LM is most consistently obtained in animals thus sensitized by hepatotoxic agents such as insecticides, antibiotics and antioxidants. The average concentration of cholesterol and total fatty acids in liver from 10 of our sensitized rats after a 24 hour fast was 3.1 and 3.7% respectively as compared with an average concentration of 0.6 and 1.1% in liver from 10 rats which had been allowed to recover for 3 weeks from exposure to the hepatotoxic agents. The average concentration of both lipids (1.9 and 2.7%) in the liver of 10 sensitized non-fasted rats was also higher than the values reported for the Wistar strain(12). These data suggest that animals treated with potential hepa-

totoxic substances either respond with greater mobilization of lipids to the liver following injection of LM, or that hepatotoxic agents interfere with utilization of mobilized fat brought to the liver. Our rats responded to a 24 hour fast by depletion of liver glycogen similar to that reported for the Wistar strain (13). The fact that hyperlipemia appeared in all the species tested indicates that the action of LM is not species or strain specific. The potency of crystalline LM, its activity in hypophysectomized rats and the failure of hypophysectomized rats to produce LM suggest the hormonal nature of this material. The increased purity resulting from crystallization was due largely to the removal of inorganic solids from the dialyzate. Preliminary studies indicate that the active material is a peptide that has neither vasopressor nor oxytotoxic actions.

Summary and conclusions. (1) The active hyperlipemia inducing constituent of the dialyzate obtained from the plasma of horses administered cortisone has been crystallized and tentatively identified as a peptide. (2) Injection of 0.02 μ g of crystalline material/kg IV induced marked hyperlipemia in intact mice, rats, guinea pigs, rabbits, dogs and humans as well as in hypophysectomized or

adrenalectomized rats. (3) The animals used in this study were exposed to potential hepatotoxic agents.

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Infectious Bovine Rhinotracheitis (IBR). I. Propagation of Virus in Cancer Cells of Human Origin (HeLa). (23255)

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Infectious bovine rhinotracheitis (IBR) virus was successfully grown in tissue cultures of bovine embryo kidney, testicle and lung by Madin and others(1), and carried by them through 37 serial transfers in kidney tissue. These authors, however obtained no indication of cytopathogenic action of the virus on HeLa, KB or L cells or chicken fibroblasts. The present communication reports propagation of IBR virus in HeLa cell cultures through 26 serial passages.

Materials and methods. *Virus strain:* The virus used in this study was obtained from Drs. J. A. Baker and J. H. Gillespie, Veterinary Virus Research Inst., Cornell University, Ithaca, N. Y., to whom we are grateful. It was received in this laboratory as a frozen lung specimen from an experimentally infected calf, sacrificed while showing symptoms of IBR. *Tissue cultures:* Trypsinized stationary tube cultures of bovine embryo kidney, lung and skin-muscle were prepared

TABLE I. Representative Titers of IBR Virus at Various HeLa Passage Levels in Bovine Kidney and HeLa Cells.

Titration in:	HeLa passage level: TC-ID ₅₀ titers							
	6	7	8	9	10	15	23	25
HeLa	10 ^{-4.5}	10 ^{-8.5}	N.T.*	10 ^{-4.5}	N.T.	N.T.	10 ^{-2.5}	10 ^{-4.5}
Bovine kidney	10 ^{-5.5}	10 ^{-6.0}	10 ^{-5.5}	10 ^{-6.0}	10 ^{-5.0}	10 ^{-5.0}	>10 ^{-4.5}	10 ^{-5.0}

* Not tested.

the sake of clarity, this is given diagrammatically in Chart 1. The original material, 20% calf lung suspension in phosphate buffer solution, was inoculated simultaneously into bovine embryo lung and skin-muscle cultures. A cytopathogenic effect became evident within 24 hours in both types of cultures and most cells had sloughed off the glass by the 3rd day. A second passage in bovine kidney cells was ready for harvest in 48 hours, whereas a third, in bovine lung and skin-muscle cultures, was again ready and harvested in 3 days. A pool of this latter passage was used to initiate the adaptation of the virus to HeLa cells. Upon the very first HeLa passage, the cells rounded and clumped up within 24 hours and, by the 3rd day, had all fallen off the glass. In the 2nd HeLa passage, however, very little change was observed in 4 days although virus was readily recovered by subinoculation into bovine kidney. A 3rd straight transfer showed no cellular alteration whatever in 7 days and was discarded. From the bovine kidney subculture fluid, essentially alternating passages between HeLa and bovine kidney cells were made. As shown in Chart 1, one main and 2 side lines of alternating transfers were carried down beyond the point where second HeLa passages showed definite cytopathologic changes. A HeLa pool of the 3 passage lines was finally made, subcultured once more in bovine kidney cells and, from the latter, propagated through 26 serial transfers in HeLa. All but 3 of these latter passages, to be commented on below, gave a similar picture: the great majority of the cells rounded up in large clumps 24 hours after inoculation with undiluted tissue culture fluid, and the cultures sloughed off the glass by the 2nd or 3rd day.

In Chart 1, HeLa cell cultures, diagrammed above the dotted line, were renewed with Eagle's basal medium containing chick se-

rum, whereas those below were renewed with Ginsberg's fluid. This change was made more for convenience than for any specific reason, and no differences were observed in the alternating HeLa passages. Later, however, in the course of serial HeLa transfers, the 16th passage was inadvertently inoculated into cultures renewed with Eagle's basal medium containing 5% normal cow serum, and the cellular changes were slower; the cultures were not ready for harvest before the 4th day. The 18th passage, with the same renewal fluid, was still slower. It was incomplete even on the 7th day. The 19th passage showed practically no change during the observation period. This trend was promptly reversed by changing back to Ginsberg's fluid, and the virus was readily recovered from the 16th and 18th passages.

Titers of virus at various HeLa passage levels in HeLa and bovine kidney cells are shown in Table I. Whereas in the latter cells the titers are comparable to those of bovine kidney grown virus, they are lower in the former and indicate a lack of correlation between HeLa and bovine kidney titers.

Identification of HeLa grown IBR virus: Two different methods were employed: 1) direct serum-neutralization in HeLa and bovine kidney cells and 2) calf inoculation.

1. Results of serum-neutralization tests on various passage levels of HeLa grown virus (Table II) are unequivocal in bovine kidney cells. Although virus titers in HeLa cultures, in the presence of normal guinea-pig serum, seem to be somewhat lower than expected, the log neutralization indices are sufficiently important for a definite identification of the HeLa grown agent as IBR virus.

2. Sixteen normal calves were bled, divided into 4 groups of 4 and inoculated as follows: animals of the first group each received 2 ml undiluted fluid (10^{7.5}TC-ID₅₀) of the 2nd

TABLE II. Identification of HeLa Grown IBR Virus by Serum-Neutralization Method.

HeLa passage	Tested in HeLa cells			Tested in bovine kidney		
	TC-ID ₅₀ titer in presence of:			TC-ID ₅₀ titer in presence of:		
	Immune G.P. serum	Normal G.P. serum	Log neut. index	Immune G.P. serum	Normal G.P. serum	Log neut. index
4	< und.*	10 ^{-1.5}	> 10 ^{1.5}			
8	"	10 ^{-2.5}	> 10 ^{2.5}			
12	< 10 ⁻¹	10 ^{-3.5}	"			
17	< und.	10 ^{-2.5}	"	< 10 ⁻¹	10 ^{-5.0}	> 10 ^{4.0}
24				"	10 ^{-4.5}	> 10 ^{3.5}

* Undiluted tissue culture fluid.

bovine kidney passage virus intramuscularly; those of the 2nd, 2 ml of the 10th HeLa passage (10^{6.2}TC-ID₅₀) also intramuscularly; animals of the 3rd group were each sprayed intranasally with 4 ml undiluted fluid (10^{7.5}TC-ID₅₀) of the bovine kidney grown virus by means of a De Vilbiss 40 nebulizer* under constant 15 lb dry nitrogen pressure, and those of the 4th were similarly sprayed with 4 ml of 10th HeLa passage virus (10^{6.5}TC-ID₅₀).

Blood specimens were again secured from all animals 2 and 4 weeks after inoculation. At the time of the 4-week bleeding, 4 normal calves were also bled. Inoculated and control animals were then challenged intranasally by the method described above, each receiving approximately 10^{7.8}TC-ID₅₀ of 2nd passage virus in bovine kidney tissue culture. A last blood specimen was taken from all calves 2 weeks after challenge. Febrile responses following first and challenge inoculations are included in Table III.

As had been observed repeatedly by 2 of the authors,[†] reaction to bovine kidney virus was far more severe in the animals exposed by the intranasal than in those infected by the intramuscular route. With the HeLa-grown virus, reactions were generally milder, even following intranasal spray. But despite differences in febrile response, animals of all groups were found to be immune on challenge, whereas the temperature curves for the 4 control calves were typical of those recorded in cases of IBR.

Observations for signs of disease were made over a period of 42 days and are also sum-

marized in Table III. Results of serum-neutralization tests on pre- and post-inoculation specimens are given in Table IV. It is of interest to note that, despite negative past histories, 2 of the animals were found to have appreciable levels of IBR neutralizing antibodies before inoculation and one remained normal despite intranasal spraying with virulent bovine kidney virus. However, it is readily observed that antibody-free animals of both intramuscular groups remained symptomless and promptly developed antibodies following live virus administration. Those receiving the bovine kidney virus intranasally underwent the classical experimental disease, recovered and developed antibodies. Symptoms were much less severe or non-existent in the HeLa-intranasal group, save for one animal which suffered a chronic form of infection, probably caused by secondary bacterial invaders, but which ultimately recovered and also developed antibodies. Antibody levels decreased somewhat from the 2nd to the 4th week after inoculation in the intramuscular group and not in the intranasal animals, but were promptly boosted in the former after challenge.

Discussion. IBR virus was adapted to grow serially in HeLa cells only after repeated alternating cycles between HeLa and bovine kidney cultures. In retrospect, this adaptation might have been accomplished by a less devious route, possibly soon after second HeLa passages showed a cytopathogenic effect. Once adapted, the virus maintained the fast destructive action it exerted on the cells upon first passage and yielded titers comparable to those of virus grown in bovine kidney when titrated in bovine kidney cells. Titers were lower in HeLa cultures. There is no

* De Vilbiss Co.

† Unpublished data.

of ACTH. The latter explanation is supported by the observation that this drug increases production of adrenal steroids in the intact animal both with normal, and with high levels of circulating adrenal steroids(6). The observations reported here might, on the other hand, be the result of an enhancement of the protein catabolic effects of the adrenal steroids by chlorpromazine. In support of a peripheral synergistic or additive action of chlorpromazine and cortisone, the findings of DeBias *et al.*, may be quoted; they found that doses of cortisone too small to protect completely a stressed, adrenalectomized rat, are much more effective when combined with subeffective doses of chlorpromazine(9).

Summary. Chlorpromazine at a dose level of 3.4 mg daily causes an increase of urinary nitrogen excretion and a decrease of weight gain in the intact tube fed rat. These changes are statistically significant. The protein cata-

bolic effect of 5 mg of cortisone acetate administered daily is increased significantly by simultaneous treatment with chlorpromazine.

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Further Studies on Release of Serotonin and Histamine During Anaphylaxis in the Rabbit. (23258)

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During anaphylaxis in the rabbit, there is a transient increase in plasma concentrations of both serotonin (5-hydroxytryptamine) and histamine(1). The increase occurs immediately after intravenous injection of the antigen, and indicates release of these amines from a bound to a free form within the animal. When an antigen is added to the whole blood of a rabbit previously sensitized to this antigen, concentrations of serotonin and histamine also increase in the plasma(1). In rabbit blood both serotonin and histamine are found almost exclusively in the platelets (2). Therefore, the rise in plasma concentrations of these amines, during the *in vitro* antigen-antibody reaction, must be due to their release from platelets. However, serotonin and histamine are found in a variety of

rabbit tissues in addition to the blood platelets. The major portion of the body serotonin is found in the gastrointestinal tract; a much smaller amount is found in the lung(3) and the brain(4). Consequently, during *in vivo* anaphylaxis, the increase of serotonin and histamine in the plasma could be due to their release either from the platelets or tissues, or from both sites. The *in vitro* study, with whole blood, suggests that at least some of the free serotonin and histamine is derived from platelets. *In vitro* studies have shown that histamine is released when organs of sensitized rabbits are perfused with a solution of the specific antigen(5). Thus, it appears that in the rabbit histamine may be released not only from platelets but also from tissues, during anaphylaxis. Similar *in vitro* studies

have not been reported for serotonin. Whether in the living animal, during anaphylaxis, serotonin is released only from platelets, or from both platelets and tissues is not known. The present study is designed to help answer this question by using reserpine in sensitized rabbits to deplete their platelets of serotonin and histamine prior to the anaphylactic reaction.

Materials and methods. Rabbits were sensitized to horse serum by intraperitoneal injection of one ml of serum every day for 6 successive days. They were used in this study when their antibody complement fixation titers were high. The rabbits that were treated with reserpine were given the drug intravenously 19 hours prior to injection of the antigen. All the animals were anesthetized with nembutal and ether. Blood was obtained by means of a catheter inserted into a carotid artery, and collected in siliconized tubes. Heparin was used to prevent clotting. Plasma serotonin and histamine, and whole blood serotonin and histamine were determined as described previously(1). Serotonin and histamine were analyzed in tissues, which had been homogenized with 0.1 N HCl, by the same procedure used for whole blood. Standards, recoveries, and blanks were run with each series of determinations. Recovery of added serotonin and histamine from plasma is essentially quantitative with these methods; from whole blood and tissues recoveries are about 80 to 95%. Details of the analytical methods are being published in a separate communication.

Results. Reserpine, in a single intravenous injection of 5 mg/kg, causes a release of most of the serotonin from rabbit platelets and tissues within 19 hours(6). Histamine is also liberated from platelets(7) by reserpine but not in a significant quantity from rabbit tissues. In this investigation, it has been found that by reducing the amount of reserpine to 0.1 mg/kg, the platelets are depleted of serotonin and histamine to essentially the same level as by the larger amount of the drug. However, in contrast, the total concentration of serotonin in the tissues is not lowered appreciably by the smaller amount of reserpine. Thus, by injecting 0.1 mg/kg of

TABLE I. Amount of Serotonin and Histamine in Rabbit Whole Blood and Intestines 19 Hr after Reserpine as Compared to Control Values from Untreated Animals. Experimental values represent range or avg of at least 4 animals. (Range was used where the analyses differed by more than 1 γ /ml or g.)

	Serotonin (γ /ml or g)		Histamine (γ /ml or g)	
	Whole blood	Intestine	Whole blood	Intestine
Normal	3-5	6.5-16	2-4	4 -7
Reserpine, 5 mg/kg	.5	3*	.8	4.5-6
Reserpine, .1 mg/kg	.7	6.5-12	.7	

* Pletscher, A. *et al.*, *Science*, 1955, v122, 374.

reserpine intravenously into rabbits, sensitized to horse serum, their platelets could be depleted of serotonin and histamine without reducing the tissue content of these amines to any great extent. Therefore, any significant increase in the plasma concentration of serotonin and histamine during anaphylaxis in such an animal should be due to a release of these amines from tissues.

The intestines in addition to the platelets are the principal source of serotonin in the rabbit(6). The amount of serotonin in the intestines far exceeds that found in any other organ, both as to total amount and per gram of tissue. Therefore, if any significant amount of the free serotonin in the plasma during anaphylaxis is derived from a tissue site, that site would be, most likely, the intestinal tract. As shown in Table I, the quantity of serotonin per gram of small intestine is high. However, even if a substantial release of serotonin occurs from the intestinal tract during anaphylaxis, it would be difficult to detect because of the wide normal variation (6.5-16 γ /g). Actual analyses of the intestines from 4 rabbits after anaphylaxis have shown that the amount of both serotonin and histamine is within the normal range. Previous work by Rose(8) revealed that the amounts of histamine in a variety of rabbit tissues following acute anaphylactic shock were not appreciably different from the amounts found in the same tissues of unsensitized control animals.

Table I gives the values for concentration

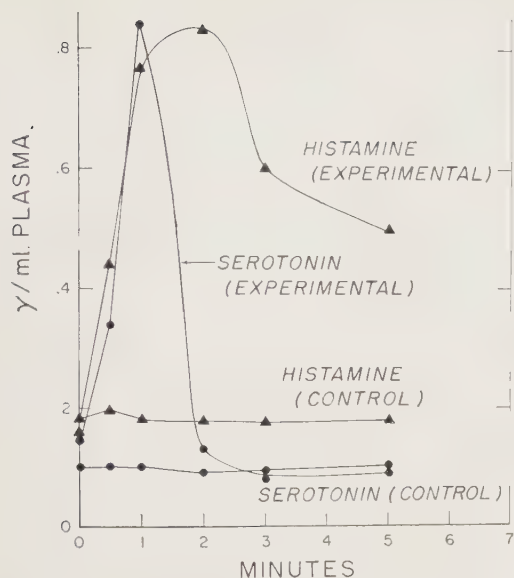


FIG. 1. Plasma serotonin and histamine in an unsensitized control rabbit and a sensitized rabbit following intrav. inj. of horse serum (2 ml/kg).

of serotonin and histamine in rabbit blood and small intestine 19 hours after the intravenous injection of either 0.1 mg/kg or 5 mg/kg of reserpine. The data reveal that the smaller amount of reserpine lowers blood serotonin and histamine to essentially the same level as the larger amount of the drug. However, 5 mg/kg of reserpine reduces the intestinal level for serotonin but not for histamine, whereas 0.1 mg/kg of reserpine does not lower the serotonin content of the intestines below the normal range.

Fig. 1 shows the increase in plasma levels for serotonin and histamine in a sensitized animal during anaphylaxis, following intravenous injection of 2 ml/kg of horse serum. The curves are similar to those shown previously(1). Unsensitized animals did not show an elevated plasma level for these amines after injection of the same amount of antigen.

Fig. 2 shows average plasma values for serotonin and histamine during anaphylaxis in rabbits that had been given 0.1 mg/kg of reserpine prior to injection of horse serum. The curves demonstrate that in comparison to Fig. 1, an increase in histamine still occurred. In contrast, serotonin levels were no longer elevated following reserpine.

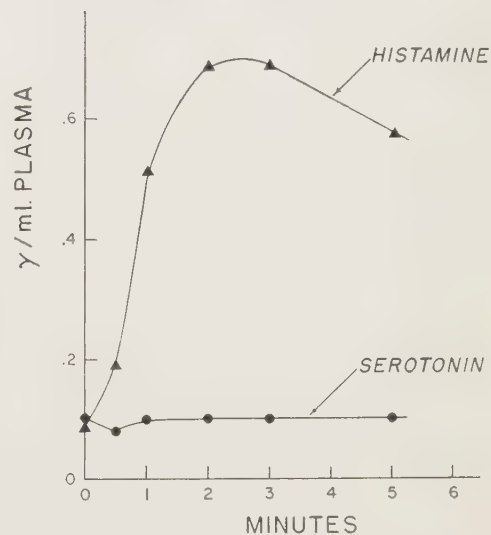
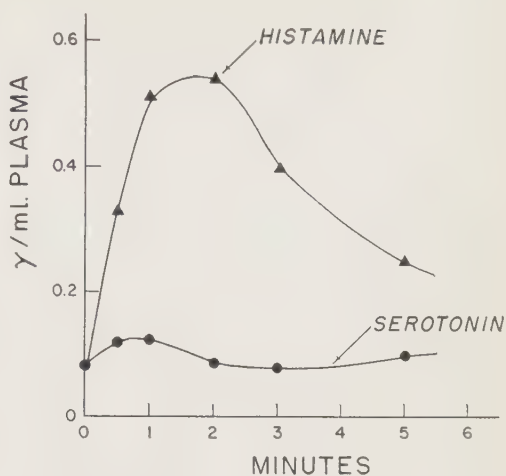


FIG. 2 (top). Plasma serotonin and histamine during anaphylaxis in rabbits that had received 0.1 mg/kg of reserpine 19 hr prior to inj. of the antigen (2 ml/kg of horse serum). Values presented are avg obtained from experiments on 3 animals.

FIG. 3 (bottom). Plasma serotonin and histamine during anaphylaxis in rabbits that had received 5 mg/kg of reserpine 19 hr prior to inj. of the antigen (2 ml/kg of horse serum). Values presented are avg obtained from experiments on 2 animals.

Fig. 3 gives average plasma values for serotonin and histamine during anaphylaxis in sensitized rabbits that had been given 5 mg/kg of reserpine before injection of horse serum. The curves are similar to those shown in Fig. 2.

Unsensitized animals, pretreated with reser-

pine in the same manner, did not show any increase in plasma concentration of serotonin and histamine after injection of horse serum.

Discussion. It appears that the major portion of the free serotonin found in rabbit plasma during anaphylaxis is probably released from platelets. On the other hand, appreciable amounts of histamine are liberated from tissues as well as from platelets following antigen-antibody interaction. However, despite these findings, it may well be that substantial amounts of serotonin could be liberated from tissues without the release being reflected by a rise in plasma serotonin. Previous work(1) showed that as much as 50 γ /kg of serotonin have been administered to normal rabbits by rapid intravenous injection without causing a rise in plasma concentration of this amine. In order to increase plasma levels of serotonin to those found during anaphylaxis, it was necessary to inject at least 150 γ /kg into a normal rabbit, whereas only 50 γ /kg of histamine were required to attain the levels found for this amine during anaphylaxis. These findings suggest that much more serotonin than histamine must be released during anaphylaxis to produce a comparable change in its plasma level, and that appreciable quantities of serotonin may be released from the tissues without producing a detectable rise in the plasma serotonin level.

Although most of the serotonin found in the rabbit is located in the intestinal tract, serotonin is also found in the lung (2-4 γ /g), and brain (0.4-0.7 γ /g). However, if the total amount of serotonin in these latter tissues were completely released during anaphylaxis, it is unlikely that the plasma serotonin would increase appreciably. Nevertheless, if only a fraction of the total lung and brain serotonin were released within these vital organs, the local effects could be profound. To consider that the relative importance of any substance, released during anaphylaxis, is demonstrated by the rise in its plasma concentration might be incorrect.

Further *in vivo* and *in vitro* tissue studies

are being conducted with rabbits to clarify, if possible, the importance of the role of serotonin in anaphylaxis. In addition, similar studies are being conducted in other laboratory animals. In most animals, the serotonin content of platelets is much less than in rabbits. If future studies prove that serotonin is not released from tissues, but only from platelets, then the importance of this amine in anaphylaxis may be less in those species where the platelet content of serotonin is low.

Summary. 1. Reserpine injected intravenously into rabbits in the amount of 0.1 mg/kg causes a marked lowering of platelet serotonin and histamine without reducing serotonin or histamine in the intestinal tract below the normal range. 2. Sensitized rabbits, pretreated with 0.1 mg/kg of reserpine, had an elevated plasma level of histamine but not of serotonin during anaphylaxis. These findings suggest that the major portion of the rise in plasma serotonin during anaphylaxis is secondary to a release from platelets, whereas the histamine found in the plasma is probably released both from platelets and tissues. In rabbits that had been pretreated with 5 mg/kg of reserpine, histamine levels in the plasma during anaphylaxis were still elevated. 3. The significance of levels attained in the plasma as an index of release from tissues during anaphylaxis is discussed.

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Effect of Hydrocortisone on C¹⁴OOH—P-Aminosalicylic Acid Distribution in Cartilage of the Rat.* (23259)

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The current practice of using salicylates to treat numerous rheumatic disorders is widespread and includes the use of hydrocortisone and similar steroids in combination with salicylates. Thus we became interested in studying the distribution of salicylates, specifically p-aminosalicylic (C¹⁴OOH-PAS) in the rat and the effect of hydrocortisone (HC) on concentration of C¹⁴ in the cartilage, following intraperitoneal injection of C¹⁴OOH-PAS. Previous workers(1) have reported that PAS is widely distributed in soft tissues of the normal rat following intraperitoneal injection of C¹⁴OOH-PAS. Studies on the fate of C¹⁴ carboxyl labeled PAS have shown that this drug is not significantly decarboxylated when administered interperitoneally(2), indicating that *in vivo* the carboxyl label is quite stable.

Methods. Litter-mate young male rats,[‡] normal and hypophysectomized, served as test animals. Normal animals, weighing about 100 g, were given intraperitoneally 5 million counts of C¹⁴OOH-PAS[§] in saline/100 g body weight. They were etherized at 30, 60 and 240 minute intervals. Tissues were removed, cleaned, weighed and homogenized in ground glass grinder. The tissue homogenate was plated on disks, dried, weighed and radioactivity determined in a windowless gas flow counter (efficiency 49%) to 3 to 5% probable error. These values were then corrected for self absorption. The tibial cap

was removed uniformly at the metaphyseal-epiphyseal junction and represented mainly articular cartilage with a little bone. The costal cartilage was easily obtained as pure cartilage. Two animals were used in each group.

Results. The data are presented in Table I. In the hormone work, rats hypophysectomized at about the age of 25 days were used experimentally on tenth day postoperatively. They received intraperitoneally 100 μ g of hydrocortisone acetate in saline or equal volumes of isotonic saline, daily for 4 days. One group of rats received both hydrocortisone and growth hormone (GH). On the fourth day, 4 hours after the last dose of hormone solution, these rats received intraperitoneally C¹⁴OOH-PAS, 5 million counts/100 g body weight. Then the animals, 2 in each group, were etherized at 15, 30, 60 and 120 minute intervals. Tissues were then prepared as previously outlined. Experiments were repeated twice with results that were correspondingly similar. Average values of 2 animals are presented in Table II.

In the normal rat C¹⁴ was found in appreciable concentration 30 minutes after injection of C¹⁴OOH-PAS, especially in the costal cartilage, tibial cap and heart tissue (Table I). After 4 hours all tissues had insignificant levels, approximately 4 counts/minute/mg (dry weight). The costal cartilage retained

TABLE I. C¹⁴OOH-PAS in Tissues of Normal Rats.

Tissue	+ 30 min.	+ 60 min.
Counts/min./mg wet wt		
Skeletal muscle	18-16.5	15.3-13.6
Heart "	39-38.6	21.9-23
Tibial cap	112-90.7	67.5-84
Costal cartilage	86-86.0	66.0-68
Counts/min./mg dry wt		
Skeletal muscle	72- 66	61- 68
Heart "	170-168	95-100
Tibial cap	178-144	107-133
Costal cartilage	176-176	134-138

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[‡] Obtained from Hormone Assay Laboratory, Chicago, Ill.

[§] Obtained from Abbott Laboratories, N. Chicago, Ill.

TABLE II. Effect of Hydrocortisone on C¹⁴OOH-PAS in Costal Cartilage.

	Control	H + HC	H + HC + GH
Counts/min./mg wet wt			
+ 15 min.	157	57	151
+ 30	92	76	72
+ 60	68	50	44
+120	40	12.2	7.4
Counts/min./mg dry wt			
+ 15 min.	320	117	309
+ 30	188	155	147
+ 60	139	102	89
+120	82	25	15

H = hypophysectomized; HC = hydrocortisone, 100 μ g; GH = growth hormone, 100 μ g.

higher levels at one hour than did the heart tissue. These values for skeletal muscle are in good agreement with those previously reported (2).

When levels of C¹⁴ activity are considered on a dry weight basis, costal cartilage, tibia cap and cardiac muscle are almost identical. However, when C¹⁴ activity is expressed on a wet weight basis there appears to be a localization of C¹⁴ labeled drug in the cartilage, a finding which is related to the fact that the relative moisture content of these tissues is quite different. It should also be pointed out that none of the tissues used was perfused, so that levels of activity given include radioactivity in blood present in the tissues. However, the cartilage, an avascular tissue, contains no blood, thus drugs probably reach it in an ultrafiltrate from the plasma. It has been shown that a large portion of blood salicylate (50-60%) is bound to plasma proteins (3), and is therefore not readily accessible to the avascular cartilage tissue. The ratio of free salicylate in the cartilage to the free salicylate in the heart is probably much higher than indicated by the figures for C¹⁴ activity because the heart contains protein-bound salicylate in the blood while cartilage, devoid of blood, presumably contains chiefly unbound salicylate.

Hypophysectomized rats attained high concentrations of C¹⁴ in the costal cartilage within 15 minutes after receiving C¹⁴OOH-PAS (Table II). These levels gradually decreased during the 2 hour observation period following the high initial values. In the hydrocortisone-treated animals, the initially ob-

served C¹⁴ deposition at 15 minutes was appreciably below that of control animals. At no time in the 2-hour study did the C¹⁴ contained in the costal cartilage of the steroid-treated rat reach values observed in the control rats. In animals given both hydrocortisone and growth hormone, the C¹⁴ salicylate content of the costal cartilage at 15 minutes approximated that noted in the control animal. However, none of the subsequent determinations presented such results. Since growth hormone has been noted to counteract many of the inhibitory actions of hydrocortisone it may be possible, by using the proper balance of hormone levels to correct this inhibitory activity of hydrocortisone on C¹⁴ salicylate concentration in cartilage. These studies are being continued using C¹⁴OOH-acetylsalicylic acid and C¹⁴OOH-sodium salicylate.

Summary. C¹⁴ as PAS and/or its metabolites is deposited in appreciable amounts in the heart, costal cartilage and tibial cap of the normal rat following the intraperitoneal injection of C¹⁴OOH-PAS. Localization in the avascular cartilage is significant in that this salicylate is mainly protein-free and thus available for cellular metabolism. In hypophysectomized rats hydrocortisone inhibited noticeably the concentration of C¹⁴OOH-PAS in the costal cartilage of steroid-treated rats when compared to the untreated control. Growth hormone tends to counteract this inhibitory action of hydrocortisone in the deposition of C¹⁴ noted 15 minutes, but not at longer intervals, after intraperitoneal injection of C¹⁴OOH-PAS. This investigation indicates that salicylate-C¹⁴ can localize in cartilage, both articular and costal, and that this concentration in the costal cartilage is decreased markedly by hydrocortisone treatment.

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Anoxic Tolerance of Beating and Resting Heart During Perfusion at Various Temperatures. (23260)

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The use of pump-oxygenators has brought such a rapid and vigorous advance of open cardiac surgery that in some instances successful clinical experiences outdistanced experimental observations. This seems to hold true for the degree of complete ischemia to which by-passed human hearts have been subjected and successfully resuscitated, although isolated dog hearts dilate rapidly when oxygen saturation of the arterial blood falls to 45% (1). The large coronary flow produced by efficient pump-oxygenators (2,3) defeats to some extent the purpose of the "bloodless heart." Therefore, attempts were made to stem the coronary tide by aortic occlusion for three (3), six (4) and even 25 minutes (5) or intermittently (6). The present study endeavors to determine the time limit of myocardial anoxia during perfusion experiments.

Methods. In heparinized dogs (3 mg/kg) pretreated with Quinidine (30 mg/kg slow i.v. drip) venous blood from the caval system was shunted by a finger pump* into a plastic chamber (Fig. 1) where it was oxygenated by bubbles, defoamed by a methylpolysiloxane† resin (7) and returned into the aorta. The volume of this oxygenator is 300 ml and its capacity tested in dogs is one l/min. A refrigeration coil replaces most of the volume and its surface serves to defoam and to cool and rewarm the oxygenated blood (8). The entire extracorporeal circuit is filled with mammalian Ringer's solution and circulation, oxygenation and cooling of the diluted blood is started simultaneously. After 15 to 30 minutes of differential hypothermia (9) the temperature in the deep oesophagus reaches the desired degree and the ascending aorta is clamped. In another series of experiments one to 2 ml of 25% potassium citrate (10) was injected into the coronary circulation to pro-

duce cardiac arrest. After the coronary occlusion period the heart rhythm, force of contraction and ability to maintain adequate blood pressure during and after extracorporeal circulation were observed for several hours.

Results. In 29 experiments in which the by-passed, but beating hypothermic heart was deprived of oxygenated blood (Fig. 2) the periods of aortic occlusion ranged from 4 to 33 minutes at temperatures ranging from 30 to 18°C. Occlusion of 25 minutes at 30°C, and for 15 minutes at 28°C proved injurious to the myocardium. When the clamp was removed these hearts fibrillated and although electric shock reestablished rhythmic contractions while their work load was carried by the pump-oxygenator, they reverted to ventricular fibrillation when the instrument was stopped and cardiac massage and repeated shocks were unable to overcome this condition. At temperatures from 26 to 20°C myocardial ischemia up to 33 minutes duration was tolerated without ventricular fibrillation or myocardial weakness. Although these periods of occlusion would be satisfactory for many intracardiac surgical procedures, they were too short to test the limits of anoxic tolerance. Ventricular fibrillation occurred in 3 cases but temperature or duration of ischemia did not seem to cause it as much as manipulation of the heart. Defibrillation was readily achieved and no ensuing myocardial weakness was discernible.

In 56 dogs the internal body temperature, as measured in the deep oesophagus, was lowered from 36° to 14°C and after clamping of the ascending aorta potassium citrate was injected into the coronary circulation to produce asystole ranging in duration from 14 to 77 minutes (Fig. 3). At 36°C an occlusion period of 27 minutes caused myocardial damage, whereas at 20°C one hour of ischemia

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† Antifoam A, Dow-Corning Co., Midland, Mich.

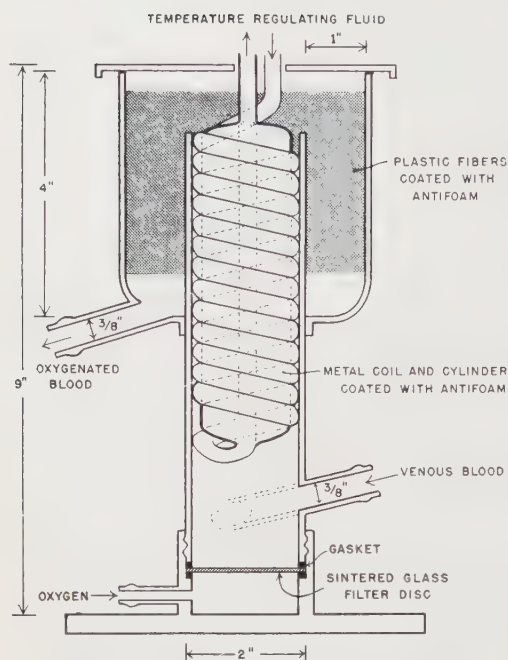
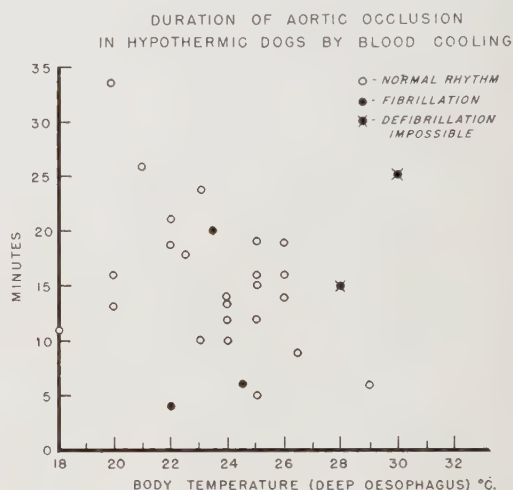


FIG. 1. Diagram of blood oxygenator.

was tolerated without ill effect. Many points indicating recovery or failure are very close and many more experiments at temperatures of 25°C and below 20°C would be needed to establish a rule, instead of just giving fairly well defined ranges of tolerance of the perfused, hypothermic, resting heart to complete ischemia. In 6 experiments ventricular fibrillation was observed during rewarming, but neither temperature nor duration of ischemia seemed to be related to it and sinus rhythm could be easily reestablished.

Discussion. These experiments were undertaken to explore the conditions under which open operations in the left side of the heart could be undertaken without the present limitation in time due to myocardial anoxia. Assuming that thorough exploration of the anatomy, delicate plastic surgery or excision of a nonfunctioning valve, choice of an eventual prosthesis, evacuation of air and closure of the incision may last one hour or longer, the aim of these and of previous investigations was to establish the essential prerequisites for such a procedure: occlusion of the ascending aorta to stop coronary flow and a myocardium undamaged by anoxia. Extracorporeal

circulation and oxygenation at normal body temperature fulfills these requirements up to 25 minutes(5), whereas slow recovery and poor cardiac action follows coronary occlusion for 40 minutes(11). If myocardial metabolism and heart rate are reduced by blood-cooling to 21°C the coronary flow can be safely stopped for at least 25 minutes(12) and for at least one hour during asystole at about the same temperature as the present study shows. Prolonged exposure of the



DURATION OF AORTIC OCCLUSION AND CARDIAC ARREST IN HYPOTHERMIC DOGS BY BLOOD-COOLING

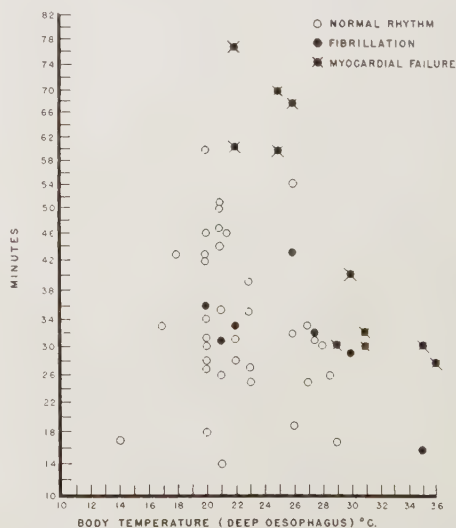


FIG. 2 (top). Anoxic tolerance of by-passed, hypothermic and contracting hearts.

FIG. 3 (bottom). Anoxic tolerance of by-passed, hypothermic and resting hearts.

mitral valve can also be obtained by hypothermic asystole at about 10°C at a very reduced systemic and coronary rate of blood flow(13) and because of the continuous oxygenation of the heart and all other organs left cardiectomy is not limited in time. For open surgery on the aortic valves the coronary flow has to be discontinued and asystole during hypothermia of about 20°C by cooling of oxygenated blood appears to be a desirable condition.

Summary. Hypothermia was produced in dogs by circulating, oxygenating and refrigerating venous blood in a small, plastic pump-oxygenator. The by-passed, contracting, hypothermic heart could be safely deprived of coronary flow for 15 minutes at 28°C and for at least 33 minutes at 20°C. During cardiac arrest induced by intracoronary injection of potassium citrate the safe period of myocardial ischemia could be prolonged to at least one hour at 20°C.

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Age Related Changes in Plasma Cholesterol of the Chicken.* (23261)

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The incidence of spontaneous atherosclerosis in the chicken is reported to increase with age(1). Since atherogenesis can be stimulated in the chick by hypercholesterolemia(2), the role of plasma cholesterol in spontaneous atherosclerosis, particularly in relation to changes of plasma cholesterol with age, becomes a matter for speculation. Data from a number of sources can be organized to suggest that plasma cholesterol increases rapidly with age in the hen. Thus 58 three-year-old hens(3) yield average value of 257 mg %, and combined data on 55 two-year-old hens (4,5) give 197 mg %. For 41 pullets between

6-9 months, grouped results(6,7,8) average 121 mg %. On the other hand, Kaishio(9) found no change in plasma cholesterol of hens between 9 and 81 months of age, but his average value of 80 mg % is considerably lower than most other published data. In males, there are no changes in plasma cholesterol up to 15 months of age(10), apparently the oldest birds studied.

Considering that plasma cholesterol is highly variable, particularly in the female, and may be affected by breed, diet, season, and reproductive state, as well as by analytical technic, it seemed desirable to restudy the problem under more uniform conditions.

Procedure. Male and female White Leg-

* Journal Series, N. J. Agri. Exp. Station, Rutgers University, State University of N. J.

horn chickens were housed on the floor and fed a standard all-mash ration. Four generations from a closed breeding flock were used, making it possible to reduce environmental effects by almost simultaneous measurements on birds varying from 1 to 5 years, as well as to make repeat measurements on some groups over years. Total plasma cholesterol was determined by the method of Zlatkis, *et al.* (11) on non-fasted animals.

Results. The results of 240 determinations on hens from 8 to 57 months old are arranged in 4 age groups in Table I. Analysis of variance indicates no significant differences among these groups, and there are no obvious changes related to age. The average plasma cholesterol for the entire series was 197 mg %.

The 149 determinations made on males from 8 to 45 months old are grouped in 5 categories in Table II. Statistical analysis indicates significant differences: in particular the 35-38 and 45 month groups differ from each other and from the 3 younger groups, but the latter are not different from one another. Thus in the male, plasma cholesterol apparently increases rapidly after the second year of life. The 209 mg % value for 45-month-old males may be questioned, since it is based on only 4 birds. This same group, however, had plasma cholesterol levels of 133 mg % at 17 months, and 158 mg % at 38 months, which are in complete agreement with average values of larger numbers at these ages.

Plasma cholesterol did not appear to be influenced to any appreciable extent by season in either sex or by reproductive state in the females.

Discussion. Our results on the hen agree with Kaishio's findings (9) to the extent that plasma cholesterol does not increase with age, although our average value of 197 mg % is

TABLE I. Relationship of Total Plasma Cholesterol to Age in the Adult White Leghorn Female Chicken.

Age (mo)	8-14	26-29	50	57
No. birds	96	103	27	14
Avg plasma chol. (mg %)	189	209	182	198
Pooled S. E. of mean*	8.9, $P > 0.05$			

* From analysis of variance.

TABLE II. Relationship of Total Plasma Cholesterol to Age in the Adult White Leghorn Male Chicken.

Age (mo)	8-12	14-16	23-25	35-38	45
No. birds	32	40	50	23	4
Avg plas. chol. (mg %)	142	143	134	155*	209*
Pooled S. E. of mean†	3.5, $P < 0.01$				

* Significantly different from all other groups. First 3 groups not significantly different from one another.

† From analysis of variance.

more than twice his. The apparent increase in plasma cholesterol of the hen with age, as derived from pooled data in the literature, may be due to differences in method, breed, diets and modes of management.

We have no explanation for the rapid increase in plasma cholesterol of the male after the second year of life. Comparison with the results of aging studies in other species is difficult. In man, the change of plasma cholesterol with age is subject to conflicting interpretations (12). It may be worthy of note, however, that in the aging rat, changes in fat metabolism occur particularly in the male (13).

These data indicate that the increase in incidence of atherosclerosis with age in the female, or in the male up to 2 years of age, is not necessarily associated with a concomitant increase in total plasma cholesterol.

Summary. The average total plasma cholesterol level of hens from 8 to 57 months old was 197 mg % and did not vary with age. The cholesterol level of males remained relatively constant at 139 mg % between 8 and 25 months, but by 35-38 months it had risen significantly to 155 mg %. At 45 months of age, a small group of males had a level of 209 mg %.

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Extractable Heparin Level in Rat Tissues Under Normal and Experimental Conditions.* (23262)

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Much of the work on the study of mast cells and their relation to the histamine and heparin content of tissues has been carried out on rats(1,2). There is, however, little available information on normal heparin levels in different rat tissues, or on properties of rat heparin. Obviously there is a need for the establishment of normal levels of extractable heparin in different organs of the rat and a comparison of the product of extraction with an accepted standard. Levels for extractable heparin have been determined for 6 different organs of the rat, and the product has been compared with beef heparin in regard to antithrombic, metachromatic and anticoagulant potencies.

Materials and methods. *Rats.* Female rats (Wistar strain) of approximately 180 g were fed a standard laboratory diet of Purina Chow. Animals were killed by decapitation, the skin immediately removed and stored at -20°C until extracted. *Cortisone.* A saline suspension of cortone acetate (cortisone acetate Merck), 25 mg/ml was given subcutaneously in daily doses of 10 mg/animal. *Carbutamide* (BZ-55), Eli Lilly and Co., Indianapolis, Ind. A solution was prepared by dissolving 4 g of the material in 100 ml of alkaline solution and adjusting pH to 7.5. Daily subcutaneous injections of 50 mg/animal were given.

Heparin extraction. The method used was

that described earlier(3) with the following additional step found desirable, when dealing with rat skin. After first alcohol precipitation the dried product was suspended in 1 ml of buffered saline/g of original tissue. The pH was adjusted to 8.0 and approximately 20 mg of trypsin/g of original tissue were added. This mixture was incubated one hour at 38°C and then treated with 80% phenol. The heparin was precipitated from the aqueous layer with 95% ethanol. For further purification the precipitate was dissolved in saline and the solution treated with benzidine as previously described(3). *Heparin assay.* (a) *Antithrombin Assay.* This was carried out according to the method of Jagues and Charles (4). (b) *Metachromatic Assay.* This was carried out as described by Jagues, Monkhouse and Stewart(5). (c) *Anticoagulant Assay.* Blood was withdrawn from a normal healthy dog by a siliconed syringe and needle and immediately placed in test tubes containing varying amounts of standard and unknown heparin solutions. The clotting times were noted and the potency of the unknown then expressed in terms of the standard heparin. *Heparin standard.* All values are expressed in terms of beef heparin kindly supplied by the Connaught Medical Research Laboratories. Unless otherwise stated the values given in Tables refer to those obtained by antithrombin assay.

Results. *Normal extractable heparin levels in rat tissue.* In Table I are shown the aver-

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TABLE I. Extractable Heparin Levels in Rat Tissues.

Organ	Animals	Units of heparin/g tissue		
		I	II	III
Heart	22	.4- .8	.4- .7	.4- .5
Spleen	22	.4- .6	.20	.24
Lung	22	.2- .3	1.4-2.4	1.9-2.3
Kidney	22	1.0-1.1	1.7-1.8	1.4
Liver	3		.7- .8	.5
Skin	42			3.9-7.8

I, Assayed after KSCN extraction, phenol treatment and alcohol ppt.

II, Assayed after treatment of product I with trypsin.

III, Assayed after benzidine treatment of II.

age levels of extractable heparin in 6 different organs of the rat. The values for skin represent results from experiments carried out over 3 months. Heart, liver and spleen show extremely low levels, lung and kidney somewhat higher and skin an average level approaching that for beef lung. The relatively high level of extractable heparin in rat skin makes this a useful tissue for the study of changes following hormone therapy or injection of histamine and heparin releasing compounds.

Table II shows results of comparison between antithrombic, metachromatic and anticoagulant activities of partially purified material from a large quantity of rat skin. Judging from preliminary measurements made by Dr. L. B. Jaques (personal communications) on recently crystallized rat heparin, our extract contains considerable amounts of inert material. Nevertheless it is of sufficient purity to indicate that we are working with a potent heparin. The low ratio of anticoagulant to metachromatic activity shows that rat heparin has a lower specific anticoagulant activity than beef or dog heparin.

Effect of cortisone and carbutamide on extractable heparin levels in rat skin. In 2 experiments the skin was obtained from rats

TABLE III. Extractable Heparin Level in Rat Skin following Cortisone and BZ-55 Treatment.

Treatment	Heparin, units/g tissue*			
	Days of treatment			
	6	9	13	15
Normal	7.1	7.0	6.5	7.8
Cortisone	7.1	3.0	5.8	7.5
Carbutamide			8.3	7.8
Cortisone + carbutamide			3.1	3.7

* Each value given is the mean of duplicate samples taken from a pool of skin from 5 rats.

which had been treated with agents affecting carbohydrate metabolism. In the first series the animals were divided into 4 groups. One group received saline injections, a second group received 10 mg cortisone daily, a third group received 50 mg carbutamide/day and a fourth group received 10 mg of cortisone plus 50 mg of carbutamide daily. On 6th, 9th, 13th and 15th days of treatment 5 animals from each of the first 2 groups were sacrificed. In addition 5 animals from each of the other 2 groups were sacrificed on 13th and 15th days. Skin from rats of each group was chopped up and thoroughly mixed. Duplicate samples of 6 g each were then taken from these pools for heparin extraction. The results are shown in Table III. The extractable heparin levels were lower in samples of skin from the cortisone treated animals than in samples from controls on the 9th and 13th days of treatment but were within the control range on the 6th and 15th days. The heparin level in samples of skin from animals injected with carbutamide only, was within the control range on both the 13th and 15th days of treatment. It was significantly lower than the control level, in the skin of rats treated with cortisone plus carbutamide, on the 13th and 15th days.

In the second experiment all animals were sacrificed after 15 days of treatment. The

TABLE II. Heparin Activity/mg of Extract from Rat Skin at Different Stages of Purification.

Stage purification	Wt of ppt (mg)	Units of heparin					
		Antithrombin		Metachromatic		Anticoagulant	
		Total	U/mg	Total	U/mg	Total	U/mg
*Benzidine precipitation	300	1200	4.0				
2nd trypsin digest	130	1000	7.7				
2nd benzidine precipitation	49.8	650	13.0	1500	30	600	12

* This represents extract from 260 g of skin from 45 different rats.

TABLE IV. Extractable Heparin Level in Rat Skin after Cortisone and Cortisone plus Carbutamide. Six animals/series.

Treatment	No. of determination	Heparin, units/g tissue
Controls	12	4.2 S = .4
Cortisone	12	2.7 S = .4
Cortisone + carbutamide	12	1.4 S = .8

$$S = \sqrt{\frac{(\bar{x} - \bar{\bar{x}})^2}{n-1}}, \text{ where } n = \text{No. determinations.}$$

heparin level was determined in duplicate for each animal. Three groups of 6 rats to a group were used. One group served as controls and received saline injection, a second group received cortisone and a third group received cortisone plus carbutamide. A summary of the results is shown in Table IV. The extractable heparin level in rats receiving both cortisone and carbutamide is in turn significantly lower than for the animals receiving cortisone only.

The results of these experiments clearly indicate that, while carbutamide alone has no effect on the extractable heparin level in rat skin, it has a significant lowering effect when given in combination with cortisone. Cortisone treatment alone caused a decrease in the extractable heparin level in both instances. However, in the first experiment the level appeared to return to normal by 15th day of treatment while in the second experiment it was significantly lower than the controls at this time.

A third experiment was therefore carried out. Twelve rats served as controls and 12 were given daily injections of cortisone. On each of the 3rd, 6th, 9th and 15th days of treatment, 3 animals from each group were sacrificed. The extractable heparin level was determined in duplicate on the skin of each animal. A summary of results is shown in Table V. It can be seen that the heparin level in the skin of animals receiving cortisone is significantly less than that of controls as early as the third day of treatment and remains low throughout the fifteen day period.

Discussion. Estimation of extractable heparin has revealed that, with the exception of skin, most rat tissues have a relatively low heparin level. Rat skin contains several times

as much heparin as any other rat tissue. This is not surprising since it also contains an abundance of mast cells. Marx *et al.*(6) recently reported values for some rat tissues which were much higher than those presented here. They suggested that their method probably gives a more complete extraction of heparin. This is certainly possible but they may also be measuring something which is not heparin. Their values for rat kidney are approximately equal to those found for dog liver and beef lung by the method of extraction used here. It would be interesting to know what levels they would obtain from these tissues with their method. They found that rat kidney had a very low mast cell count, despite the high heparin content. It is quite consistent with present-day knowledge to find a tissue with a high concentration of metachromatic staining cells and a low heparin content, since other sulphonated polysaccharides will stain similarly. It remains to be explained, however, how a compound such as heparin can be present in large amounts without showing the characteristic metachromatic reaction. These authors do not report values for rat skin.

It was recently shown that the amount of extractable heparin in the liver of hypophysectomized dogs was greater than in control animals(3). This finding, coupled with reports(7) that the injection of cortisone or ACTH causes a decrease in the number of mast cells in dermal connective tissue of the rat suggests that a metabolic pathway involving heparin is influenced by the pituitary gland through the adrenal cortex. The reduc-

TABLE V. Extractable Heparin Level in Rat Skin during Cortisone Treatment.

Treatment	Heparin units/g tissue			
	Days after treatment			
	3	6	9	15
Control	(3) 5.0 S = .65	(3) 4.6 S = .60	(3) 4.9 S = .65	(3) 3.9 S = .50
Cortisone	(3) 2.6 S = .30	(2) 3.4 S = 1.25	(3) 2.2 S = .82	(3) 2.7 S = .35

() = No. of animals; duplicate determination carried out on each animal.

$$S = \sqrt{\frac{(\bar{x} - \bar{\bar{x}})^2}{n-1}}, \text{ where } n = \text{No. determinations.}$$

tion in mast cells by cortisone and ACTH injections has been reported for other species (8).

The results of experiments reported here show that the extractable heparin level in rat skin is decreased by the injection of cortisone. Presumably this decreased level is due to an inhibition of heparin formation, since Layton (9) showed that cortisone inhibited mucopolysaccharide synthesis in the intact rat and Böström and Odeblad (10) have shown that cortisone decreases the rate of sulphate exchange in chondroitinsulphuric acid.

The chance finding that carbutamide augments the depressing action of cortisone on heparin level is of immediate interest. It is worth noting that Dr. D. W. Clarke of this department found, that compared to controls, the cortisone treated animals had significantly higher per cent liver fat and, that the animals treated with both cortisone and carbutamide had a higher per cent liver fat than those given cortisone only. An increase in liver fat is thus found under conditions which also produce a decreased heparin level in the skin. Further experiments will be required to determine if these are merely coincidental happenings or if they are indeed related.

Summary. The extractable heparin level

in different rat tissues has been measured. The skin of the rat is relatively high in heparin but the content of the other tissues measured is low. The heparin extracted has a lower specific anticoagulant activity than either beef or dog heparin. The skin of rats receiving daily cortisone injections has a significantly lower heparin level than that of controls. Injection of Carbutamide (BZ-55) alone into rats does not affect the extractable heparin level but it greatly augments the depressing action of cortisone.

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Effect of Intravenous Soy Bean Phosphatides on Blood Coagulation in Rabbits.* (23263)

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Inosithin® is a commercial preparation of alcohol-insoluble phosphatides derived from soy beans. This material has coagulant properties resembling those of platelets, and it functions as an effective platelet substitute in the thromboplastin generation test and in the partial thromboplastin time.† Moreover, like platelets, Inosithin® is anticoagulant *in*

vitro when concentrations above the optimal range are used (1,2). The following study was performed to determine the *in vivo* effects of Inosithin® on blood coagulation.

Methods and materials. Experimental subjects were male rabbits weighing between 2.5 and 3.5 kg. Inosithin® was administered as a 5% emulsion suspended in 5% glucose solution.‡ This emulsion gave optimal activity

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† Partial thromboplastin studies were performed by Dr. John Graham of Univ. of North Carolina.

‡ Inosithin was kindly provided by Mr. J. Eichberg of Associated Concentrates, Woodside, L. I., and prepared as a sterile emulsion by Don Baxter, Inc., Glendale, Calif.

in the thromboplastin generation test when diluted 1:100. Infusions were given into the marginal ear vein at a rate of about 5 cc per

minute. Blood was collected from the central artery of the ear using 20 gauge needles, and into untreated glassware. When plasma was

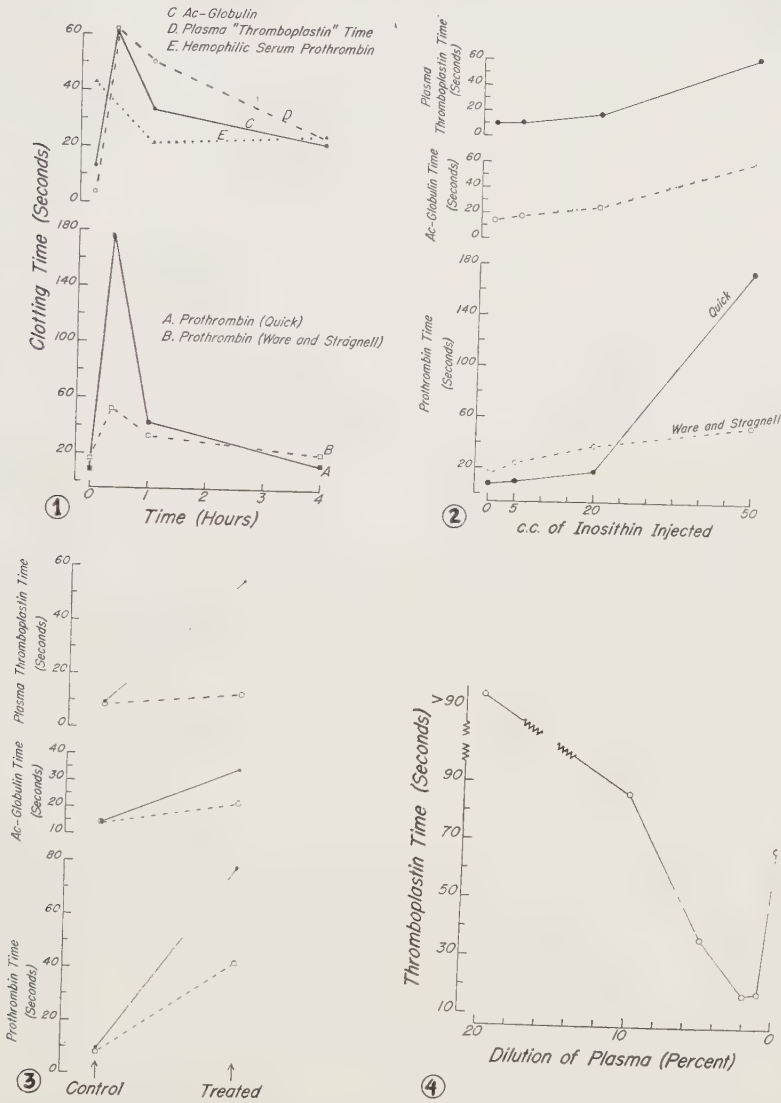


FIG. 1. Serial clotting changes in a 3 kg rabbit following 50 cc of Inosithin® given intrav. Curves A, B, and C are self-explanatory. Curve D represents clotting time in thromboplastin generation test after 6 min. of incubation, with animal's plasma used as source of AHF. Curve E is serum prothrombin time of hemophilic blood to which had been added test plasma in final conc. of 1%.

FIG. 2. Coagulation changes in rabbits given varying doses of Inosithin® intrav. See legend of Fig. 1 for explanation of terms.

FIG. 3. Comparison between *in vitro* and *in vivo* effects of Inosithin® on coagulation in rabbits. Solid line shows effect of 50 cc dose into a 3.5 kg rabbit 15 min. after completion of inj. Broken line shows effect of mixing normal rabbit plasma with $\frac{1}{4}$ volume of Inosithin® emulsion.

FIG. 4. Use of post-inj. plasma as platelet reagent in thromboplastin generation test. Plasma was taken from blood drawn 15 min. after a 50 cc dose of intrav. Inosithin®. The times shown are those obtained with normal plasma and serum reagents, and following 6 min. of incubation of the generating mixture.

required, a syringe was used containing anti-coagulant; blood for serum was allowed to drip directly into test tubes. Plasma was obtained from blood anticoagulated with one-tenth volume of 0.1 molar potassium oxalate. Coagulation procedures were performed according to methods previously described (3,4).

Results. When Inosithin® was given to the rabbits intravenously in doses up to 50 cc, no visible toxic effects were evident. Blood specimens drawn within 2 hours after injection of the maximal dose showed greatly prolonged whole blood clotting times in glass, and in lusteroid tubes clotting failed to occur even after 8 hours in some samples. In the many animals tested the coagulation response was qualitatively uniform, although there was a moderate variability in degree of coagulation changes.

Fig. 1 shows a representative study of serial changes following a 50 cc dose of Inosithin®. There was rapid and profound depression of prothrombin, Ac globulin, and AHF. These changes were most pronounced in blood samples drawn shortly after completion of the injection, and gradual restoration toward normal occurred in the subsequent 4 hours. No marked or consistent abnormality was encountered when serum from injected animals was used in the thromboplastin generation test, indicating no significant depression of plasma thromboplastin component (PTC), Factor X, or Stuart Factor. Likewise, there was no change in the plasma fibrinogen.

The degree of clotting abnormality developing in injected animals was dose-dependent. Fig. 2 shows the effect of varying the volume of injected Inosithin® from 5 to 50 cc. In each case blood specimens were collected 15 minutes after completion of the injection. Changes were relatively minor until a dose of 20 cc was reached despite the enormous amount of coagulation-active lipid transfused. Of additional interest is the greater prolongation of the Quick prothrombin as compared to the Ware and Stragnell (5) test, at maximal dose levels. This discrepancy is partly due to the provision in the Ware and Stragnell reagent of excess Ac globulin. However, as will

be shown, this is not the entire explanation.

At least part of the Inosithin® effect in the animals was independent of its direct action on the blood, for the changes induced *in vivo* differed from those resulting from *in vitro* mixing. As shown in Fig. 3, the addition of $\frac{1}{4}$ volume of Inosithin® emulsion to pre-injection rabbit plasma had a significantly lesser effect on the Quick prothrombin and Ac globulin than an *in vivo* dose of 50 cc. Moreover, the *in vitro* Inosithin® had little effect on activity of plasma in the thromboplastin generation test, indicating no direct depression of AHF. The blood Inosithin® level of the injected rabbit could have been no greater than that achieved by *in vitro* mixing, for the plasma volume was approximately 180 cc. Actually, because of some lipid clearing from the blood of the injected animal during injection and prior to collection of specimens, the Inosithin® level was probably appreciably lower than in the specimen mixed *in vitro*.

Anticoagulant activity was present in the blood of rabbits injected with the 50 cc dose of Inosithin®. Table I shows that plasma drawn 15 minutes following this dose of the lipid prolonged the prothrombin time of pre-injection plasma when these plasmas were mixed in equal amounts. This prolongation was considerably greater than that produced by simple dilution of the pre-injection plasma with isotonic saline. The anticoagulant effect was not the result of antithrombin, for the thrombin times of plasma from injected animals were identical to that of their pre-injection sample.

Coagulant activity resembling that of Inosithin® could be demonstrated in the plasma of injected rabbits. The plasma of blood drawn 15 minutes after completion of a 50 cc dose was an effective platelet substitute in the thromboplastin generation test. As shown in

TABLE I. Anticoagulant Action of Post-Injection Rabbit Plasma.

	Quick prothrombin time (sec.)
Control plasma	9.0
<i>Idem</i> + saline	11.4
Post-injection plasma	360
<i>Idem</i> + control plasma	42.4

Fig. 4, such plasma after barium sulfate adsorption effected normal thromboplastin generation when appropriately diluted, but was anticoagulant in excessive concentration. Plasma from untreated controls had no such platelet-like activity.

Discussion. The data presented above show that soy bean phosphatides with platelet-like coagulant activity have profound effects on blood coagulation when given intravenously. The findings indicate that clotting is depressed as a result of two mechanisms. The first of these is a direct anticoagulant action of highly concentrated phosphatides, shown by the ability of post-injection plasma to interfere with clotting in normal plasma. However, the greater effect of *in vivo* phosphatides over that produced by comparable concentrations added *in vitro* suggests a second mechanism: a partial conversion of prothrombin into thrombin. Evidence in favor of this view is seen in the combination of prothrombin, Ac globulin, and AHF depression together with intact levels of those clotting factors not consumed in the course of clotting. In other words, the blood of injected animals showed many properties of serum.

It is of considerable interest that the above effects were produced only by massive doses of phosphatides, and that the "small" doses had little demonstrable action. Even the 5 cc dose of Inosithin® possessed activity equaling that of platelets from an entire blood volume of the experimental subjects. Despite the intimate mixture of all elements necessary for clotting, generalized coagulation was not precipitated nor was there a demonstrable

change in fibrinogen even following the largest doses. Such findings with injected platelet products have been interpreted to mean that platelets do not initiate blood clotting under physiological conditions(5). However, an alternative hypothesis might be that intermediate products of blood coagulation are cleared from the blood as it circulates, possibly by some cellular mechanism. This would account for our observed prothrombin conversion without fibrinogen depression, and for the apparent inability of smaller amounts of Inosithin® to cause extensive blood thromboplastin formation.

Summary. Emulsions of soy bean phosphatides with platelet-like coagulant activity were given intravenously to rabbits. Small doses were followed by little change in blood coagulation, but larger doses produced depression of prothrombin, Ac globulin, and AHF. Fibrinogen and the "serum factors" were unaffected. The observed changes resulted from direct anticoagulant activity, and probably also from partial conversion of prothrombin.

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Correlation between Plasma Amylase Activity and Concentration of Non-esterified Fatty Acids (NEFA)* (23264)

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(Introduced by V. P. Dole)

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Administration of insulin, glucose or glucagon lowers plasma amylase(1-3) and causes a sharp drop in plasma non-esterified fatty acids (NEFA)(4-6). These apparently unrelated blood components have not been studied jointly, however, nor has amylase level been examined in relation to other agents known to affect NEFA concentration. In the present investigation glucagon, glucose, fructose, epinephrine, and orinase were administered to normal and diabetic patients. They were found to produce strikingly parallel variations of amylase and NEFA.

Methods. Normal and diabetic patients were studied at Mt. Sinai or Rockefeller Institute Hospital. After an overnight fast, blood samples for determination of amylase (7), glucose(8), and NEFA concentrations (4) were taken in duplicate prior to administration of a test agent and at 20-minute intervals for 2 to 4 hours thereafter.

Results. Fig. 1 shows the average percentage changes of blood sugar, amylase and NEFA in 19 non-diabetic and 9 subjects with stable diabetes following administration of glucagon. Amylase and NEFA varied in parallel ($p < .001$) ($r = 0.65 \pm 0.09$). The reductions in concentration of both components were of greater magnitude in normal subjects than in diabetics ($p < .001$ at 40 min.) and, as with the blood sugar, the returns toward control value in normals were more rapid. Glucose tolerance tests (5 normal subjects) and a fructose tolerance test (one normal subject) (Fig. 2), caused decreases of both amylase activity and NEFA concentration with reciprocal rises of blood sugar. Insulin, known to produce a fall in plasma NEFA associated with a decrease of blood sugar(4), also caused a significant reduction in amylase in 14 patients ($p < .001$). Orinase (1-butyl-

3-p-tolylsulfonylurea), 2.0 g administered intravenously to 4 stable diabetics and 4 normal subjects (Fig. 3), caused hypoglycemia with associated decreases of amylase and NEFA concentrations ($p < .001$) Epinephrine (1.0 mg) given subcutaneously (Fig. 4), produced parallel increases of both amylase and NEFA in 2 normal subjects.

Discussion. The sources and functions of blood amylase are unknown. Enzymes with amylase activity can be extracted from pancreas, salivary glands, and liver. In clinical practice elevation in blood amylase usually is attributed to regurgitation of the external pancreatic secretion as a result of duct obstruction(9). However, the fact that blood amylase is maintained following removal of the pancreas in the dog(1) and man(10), suggests that non-pancreatic sources are more important than pancreas for maintenance of normal blood levels. Both pancreas and salivary glands have been totally removed from rats without causing a decrease in blood amylase(11). By exclusion, it appears that the liver might be the main source of blood amylase(12).

At any rate, changes in blood amylase are distinct from changes in amylase content of pancreatic juice: (a) insulin hypoglycemia, which is a stimulus to exocrine secretion of pancreatic amylase(13), caused a fall in blood amylase; (b) epinephrine, which diminishes exocrine secretion of amylase by the pancreas(14), elevated blood amylase; (c) secretin and pancreozymin, which stimulate pancreatic flow and enzyme secretion in patients with a normal unobstructed pancreatic duct system, have no effect on blood amylase (9); (d) amylase and NEFA responses to glucagon in one patient following subtotal pancreatectomy were similar to the results obtained in diabetic subjects with no known defect of external pancreatic secretion.

The consistently parallel variations of blood

* The Glucagon was furnished by the Eli Lilly Co., Indianapolis, and the Orinase by Upjohn Co.

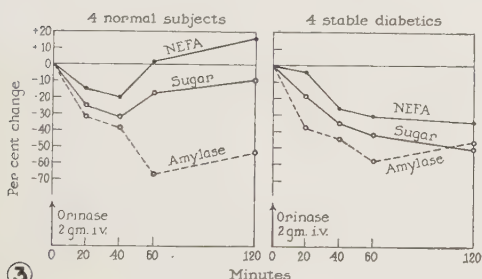
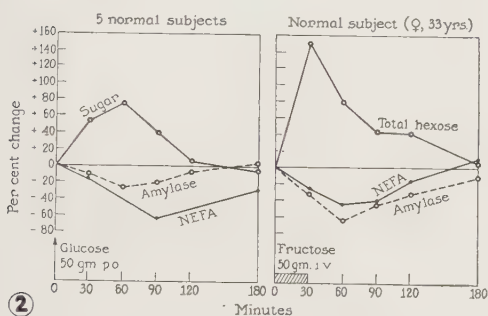
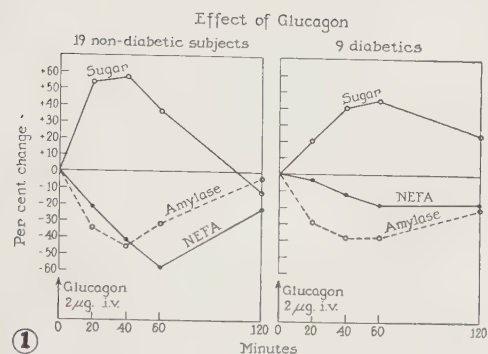


FIG. 1-3.

amylase and NEFA suggest these two components might be involved in some common process. The decrease in plasma NEFA following administration of insulin appears to be due to reduction in delivery of fatty acids from tissue stores to blood(15). Possibly the associated decrease in amylase is related to this reduced flow of fatty acids to cells. It is also interesting to note that insulin and glucose, which increase carbohydrate utilization, produced a decrease in plasma NEFA and amylase, while epinephrine, which promotes fat catabolism, caused an increase in both NEFA and amylase.

Summary. Blood amylase and plasma non-esterified fatty acid concentrations responded in a correlated way to glucagon, glucose, fruc-

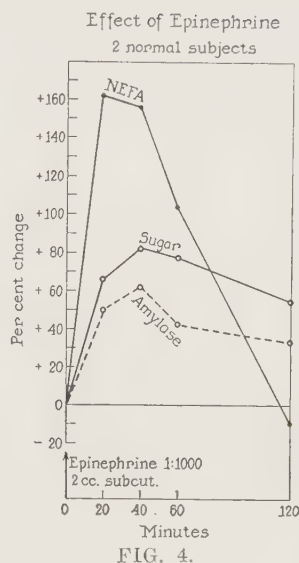


FIG. 4.

tose, epinephrine and orinase. Apparently these two components are associated in some common metabolic process, extrapancreatic in location.

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Activity of Certain Vit. B₁₂ Analogues in the Chick.* (23265)

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A number of compounds structurally related to vit. B₁₂ have been obtained by isolation from substances supporting growth of microorganisms or by chemical modification of cyanocobalamin (as reviewed in 1,2). These compounds differ in their ability to replace vit. B₁₂ in various living organisms. The simplest analogues available at present are those cobalamins in which a molecule of water or an anionic group is substituted for the cyanide radical. Rosenblum and co-workers(3,4) have compared absorption and urinary and fecal excretion of chloro-, sulfato-, nitro-, and thiocyanatocobalamin with cyanocobalamin in healthy young men. They found that absorption of the analogues was lower than that of cyanocobalamin, although all compounds appeared to be excreted similarly upon injection. In rats, poor absorption of chloro- and nitrocobalamins was also found. Vit. B₁₂ analogues synthesized by microorganisms usually differ from vit. B₁₂, by changes in the nucleotide portion of the molecule. In the case of factor B, no nucleotide is present(5). Vit. B_{12_{III}}, or factor III, has a 5-hydroxybenzimidazole moiety replacing 5,6-dimethylbenzimidazole of vit. B₁₂ (6-8). Factor B has been found to be inactive in treatment of pernicious anemia(9) and in supporting growth of chicks(9,10), whereas the 5-hydroxy analogue produced a hematological response in patients with pernicious anemia(11,12) and had about 4% of the activity of vit. B₁₂ as determined by growth of chicks(10).

In the present investigation the vit. B₁₂ activities of acetato-, chloro-, nitro-, and sulfatocobalamin, factor B, and the 5-hydroxy analogue were determined in the young

chick.

Methods. Female New Hampshire chicks from a commercial hatchery were distributed into groups of 6 chicks each and maintained on the experiment from 1 to 28 days of age. The chicks were weighed at weekly intervals; diet and water were offered *ad lib*. The chicks were fed a corn-soybean oil meal diet high in fat, which was identical to that used to evaluate the activity of desdimethyl B₁₂(13) except that 1 mg 2-methyl-1, 4-naphthoquinone was added per kg of diet. The analogues were dissolved in ethyl alcohol and appropriate aliquots were added to the diet or diluted with water for parenteral administration.

Results. The 4-week weights of chicks receiving graded levels of vit. B₁₂, acetato-, chloro-, nitro-, and sulfatocobalamin are presented in Table I. At each level of analogue, growth was similar to that with the same level of vit. B₁₂. In only one instance, that of 10 γ nitrocobalamin/kg, was a significant difference seen, and this was borderline. In none of the individual experiments was it possible to demonstrate significant differences between analogue and vitamin. In these experiments each compound elicited similar growth responses when it was injected as when it was fed at the same level in the diet.

In Table II, the activities of factor B and the 5-hydroxy analogue are compared with that of vit. B₁₂. The 5-hydroxy analogue had about one-tenth to one-third the activity of vit. B₁₂. Factor B did not significantly affect growth, although the 4-week weight of chicks receiving 10 γ factor B/kg of diet was somewhat lower than that of the basal group.

Discussion. The good growth of chicks obtained when the cobalamins were fed in the diet indicates that presence of cyanide in the vit. B₁₂ molecule is not necessary for maximal utilization in this species. These results differ from the findings of Rosenblum *et al.* (3,4), who demonstrated that more of an oral test dose of cyanocobalamin was absorbed

* The authors wish to thank Dr. Charles Rosenblum of Merck and Co. for the cobalamins and Dr. Karl Folkers of Merck and Co. for the 5-hydroxy analogue and factor B that were used. The authors gratefully acknowledge the technical assistance of Helen M. Hood and Woodrow W. Duvall.

than that of the other cobalamins they studied. It is possible that the cobalamins may be converted in the chick's gut to some form which is more easily absorbed or these differences may reflect a basic difference in the absorption mechanism of the chick as compared with man or the rat. Evidence for species specificity of intrinsic factor-binding and subsequent absorption of vit. B₁₂ has been demonstrated (14). It does not seem likely that the different experimental approaches, chick growth *vs.* the Schilling test, could account for the differences observed.

From the studies reported to date, vit. B₁₂ analogues that support growth of the chick have also been active in patients with pernicious anemia. The young chick is therefore a very useful assay organism for anti-pernicious anemia activity, since microorganisms lack this specificity of response.

Summary. Activity of various vit. B₁₂ analogues was determined in young chicks fed

TABLE I. Growth of Chicks Fed Graded Levels of Vit. B₁₂ and Some of Its Analogues (6 Chicks Started per Experimental Group).

	γ of supplement/kg of basal diet	No. of chicks*	Avg wt in g at 4 wk and S.E.
Vit. B ₁₂	0	37 (5)	167 \pm 9
	5	28 (2)	233 \pm 12
	5 inj.†	9 (3)	231 \pm 12
	10	23 (1)	248 \pm 9
	10 inj.	18 (0)	257 \pm 10
	100	42 (0)	290 \pm 6
Acetatecobalamin	10	11 (1)	255 \pm 12
	10 inj.	10 (2)	241 \pm 10
	100	18 (0)	297 \pm 10
Chlorocobalamin	5	24 (0)	236 \pm 11
	5 inj.	22 (2)	242 \pm 13
	10	23 (1)	260 \pm 9
	10 inj.	23 (1)	265 \pm 10
	100	17 (1)	286 \pm 13
Nitrocobalamin	5	22 (2)	230 \pm 12
	5 inj.	24 (0)	257 \pm 14
	10	23 (1)	284 \pm 11‡
	10 inj.	24 (0)	259 \pm 10
	100	11 (1)	284 \pm 12
Sulfatocobalamin	10	18 (0)	258 \pm 10
	10 inj.	18 (0)	282 \pm 13
	100	18 (0)	285 \pm 13

* No. in parentheses is No. of chicks that died.

† Inj. made 3 times/wk. Amount of compound inj. was equal to amt of same compound consumed during preceding 2-3 day period by group receiving indicated level in the diet.

‡ Wt significantly different ($p = <0.05$) from group receiving same amt of vit. B₁₂.

TABLE II. Growth of Chicks Fed Graded Levels of Vit. B₁₂, the 5-Hydroxy Analogue of Vit. B₁₂, and Factor B (6 Chicks Started per Experimental Group).

	γ of supplement/kg of basal diet	No. of chicks*	Avg wt in g at 4 wk and S.E.	Avg % change in wt†
Vit. B ₁₂	0	26 (4)	167 \pm 9	
	10	24 (0)	244 \pm 8	46
	10 inj.‡	12 (0)	253 \pm 8	52
	100	30 (0)	293 \pm 9	76
5-hydroxy analogue	10	21 (3)	194 \pm 11	16
	10 inj.	23 (1)	174 \pm 12	4
	100	22 (2)	229 \pm 10	37
	100 inj.	5 (1)	213 \pm 18	28
Factor B	10	9 (3)	136 \pm 13	-19
	10 inj.	9 (3)	149 \pm 12	-11
	100	11 (1)	161 \pm 13	-4
	500	5 (1)	202 \pm 7	21

* No. in parentheses is No. of chicks that died.

† Change in wt from basal group (no B₁₂).

‡ Inj. made 3 times/wk. Amount of compound inj. was equal to amt of same compound consumed during preceding 2-3 day period by group receiving indicated level in the diet.

a corn-soybean oil meal diet high in fat. The presence of cyanide in the molecule is not necessary for full vit. B₁₂ activity in the chick since acetate-, chloro-, nitro-, and sulfatocobalamin had activities equal to that of vit. B₁₂ when fed or injected. Factor B had no vit. B₁₂-like activity nor any antagonistic action. The 5-hydroxy analogue of vit. B₁₂ had about one-tenth to one-third of the activity of vit. B₁₂.

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Effects of Unsaturated Fat on Serum Lipids in Idiopathic Hyperlipemia. (23266)

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It has been known for some time that dietary fat influences serum lipid levels. Beveridge *et al.*(1) found that diets high in animal fat produced elevations in serum lipid levels, while diets high in fats of vegetable origin did not. Ahrens and his associates(2) also produced lower serum lipid levels with vegetable oils, especially corn oil. The serum lipid lowering effect of vegetable fat has been attributed by Kinsell(3) and more recently by Bronte-Stewart(4) to its higher content of unsaturated fatty acids. Bronte-Stewart(4, 5), in particular, has shown that the lipid lowering effect of administered fats in normal individuals may be directly proportional to the unsaturated fatty acid content of the diet. It was reported by Lever(6), and confirmed in this laboratory(7), that the intravenous administration of a cottonseed oil preparation (Lipomul I-V)* on some occasions led to a drop in serum lipids in patients with idiopathic hyperlipemia. Administration of the oral product (Lipomul Oral)* failed to lower serum lipids. Since the oral preparation is primarily coconut oil, containing almost no unsaturated fatty acids, this may explain the lack of success with the oral product. According to Deuel(8), the iodine number of coconut oil is 8-40, while that of cottonseed oil is 105-115.

Methods. Two patients with idiopathic hyperlipemia were placed on diets carefully

controlled with respect to fat content. One patient, E.B., was a male 29 years old, the other, D.L., a male 31 years old. Neither patient was hospitalized during the course of the experiments. The following diets were used in this study: Diet I, uncontrolled with respect to fat content; and Diet II, which contained 120 g fat consisting of 50 g of highly purified soybean oil,[†] 30 g of soy phospholipid concentrate,[‡] and 40 g animal fat. Diet III, administered to only one patient (D.L.) had no added soy phospholipid concentrate. This diet, which was isocaloric with Diet II, contained 50 g of soybean oil,[†] 40 g of animal fat, and 10 g of vegetable fat. The caloric content of all diets was 2400-2600. Serum lipid partitions were done on these patients on fasting blood samples taken

[†] Supplied by the Procter and Gamble Co., Cincinnati, O. This soybean oil had the following composition:

Iodine No.	131.9
Total fatty acids	95.9 %
Free " "	.0
OH number	1.6
Unsaponifiable	.5
Tocopherol	.12
Trans fatty acids	.2

Spectrographic analysis yielded the following values:

Saturated fatty acids	16.6 %
Oleic acid	21.2
Linoleic acid	50.4
Linolenic acid	7.7

* Supplied by the Upjohn Co., Kalamazoo, Mich.

[‡] Purchased from Associated Concentrates, Woodside, L. I., N. Y.

TABLE I. Effect of Unsaturated Fats on Serum Lipids. Patient E. B.

	Diet I*	Diet II*				Diet I*	
	10/15	(Fed 10/16-12/22)				(Fed 12/22-3/1)	
		11/3	12/3	12/21		1/31	3/21
Total lipid†	2137	1314	1219	1012		1454	1705
Total cholesterol†	560	471	302	274		448	478
% cholesterol esters	77	81	76	80		77	80
Phospholipids†	413	418	366	263		369	463
Triglycerides†	888	184	395	325		403	505

* See text for description of diets.

† Values expressed as mg %.

at intervals before, during, and after the administration of the special diets. Total lipids were determined by the method of Bragdon (9), total cholesterol and cholesterol esters by the Schoenheimer-Sperry method (10), and phospholipids by the Fiske-Subbarow method (11).

Results. The serum lipid values resulting from the present study are listed in Tables I and II. It can be seen from Table I that the first patient, E.B., showed a marked drop in serum values for total lipid, total cholesterol, phospholipid, and triglycerides during the period of administration of Diet II. When the patient resumed eating Diet I, these values all showed marked increases. Although the absolute values did change, there was no significant change in percentage of cholesterol present in esterified forms.

Table II shows the results obtained on the second patient, D.L. The same qualitative picture is repeated. The extraordinarily high total lipid values obtained on this patient under Diet I showed marked decreases during the administration of Diet II. Resumption of Diet I produced very great elevations in all serum lipid values studied. This patient was then placed on Diet III, which contained high levels of unsaturated fatty acids, but without added phospholipid concentrate.

During the period of Diet III, the patient again demonstrated marked decreases in all the serum lipid fractions studied. It should be noted that this patient also demonstrated no significant change in the percentage of cholesterol present in esterified forms. Both patients had opaque, milky sera, which cleared during the periods on Diets II and III. Both patients had tuberous xanthomata of the extensor joint surfaces. One patient, D. L., had generalized papular or eruptive xanthomas and lipemia retinalis. The papular xanthoma and lipemia retinalis disappeared, and the tuberous xanthoma diminished in this patient during the administration of Diet II. Patient E.B. showed some disappearance of the tuberous xanthomata.

Discussion. This dietary study would seem to support the concept that the lipid lowering effect of administered fats may be dependent on the unsaturated fatty acid content of these fats. The recent findings of Bronte-Stewart (4,5) suggest that unsaturated dietary fat may prevent elevations of serum lipid in the presence of moderately large amounts of dietary animal fat. We believe that the study of hyperlipemic patients provides a very convenient means of evaluating this hypothesis, because of the very high initial serum lipid values found in idiopathic

TABLE II. Effect of Unsaturated Fats on Serum Lipids. Patient D. L.

	Diet I*	Diet II*		Diet I*	Diet III*
	10/15	(Fed 10/16-12/21)		(Fed 12/21-2/1) (Fed 2/1-3/1)	
		11/21	12/20	1/31	3/1
Total lipid†	20316	1985	3627	23327	3724
Total cholesterol†		454	489	2344	582
% cholesterol esters		75	72	71	71
Phospholipids†		429	728	2104	877
Triglycerides†		871	2171	17741	1984

* See text for description of diets.

† Values expressed as mg %.

hyperlipemia.

Summary. Two patients with idiopathic hyperlipemia were placed on 2400-2600 calorie diets containing 50 g of highly purified soybean oil, with or without the addition of a phospholipid concentrate. Marked decreases in serum lipids occurred during the period of administration of both diets. The serum lipid decreases were independent of phospholipid concentration in the diet, and believed attributable to the presence of unsaturated fatty acids in the dietary fat. Clinical signs of the disease regressed or disappeared coincident with the decrease in serum lipids.

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Effect of 5-Hydroxytryptophane Upon Electroencephalogram in Hepatic Coma.* (23267)

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Liver failure is usually associated with serious disturbances of consciousness. These episodes are accompanied by electroencephalographic changes of a diffuse sort with an increase in amount and amplitude of slower frequencies and a diminution of faster frequencies. Some investigators have reported what they considered to be characteristic neurologic signs(1) and electroencephalographic patterns(2-3) in liver disease, but these have not been observed uniformly(4). Observations of increased ammonia content of blood in liver failure(5-6), caused by inability of the liver to remove or "detoxify" ammonia from the portal blood because of shunt or deficiency of the urea synthesizing system have led to an hypothesis for the mechanism of ammonia-genic coma(7). The liver, however, does far more than detoxify substances. It has been pointed out recently that some metabolites

essential to brain are probably synthesized in the liver and that liver failure may lead to serious deficiency of these substances(8). Among these may be the precursor material for serotonin, which appears to play a significant role in brain metabolism(9-11). Parenteral injection of moderate amounts of serotonin causes no change in the brain content of this intermediate, but administration of small amounts of a precursor substance, 5 hydroxytryptophane, (5HTP), does cause a large increase of serotonin in brain(12-13) together with symptoms similar to those seen after a block in serotonin metabolism in brain (14) caused by lysergic acid diethylamide. Although the synthesis of 5 HTP in liver preparations has not been demonstrated, other hydroxylations of a similar nature have occurred in that organ. Indirect evidence for failure of supply of the serotonin precursor comes from observations that urinary excretion of 5 hydroxyindole acetic acid, (5HIAA),

* Aided by Grant from the Playtex Park Research Fn.

a major terminal metabolite of serotonin, is diminished in liver disease(15).

Methods. In view of these observations 10 mg of dl 5-hydroxytryptophane were given intravenously to 3 patients with liver failure and to 3 individuals who were not in liver failure. Electroencephalograms were made before, during, and after infusion. Measurements were made of the excretion of 5 HIAA, and of blood levels of serotonin during the experiment. These will be reported elsewhere.

Results. The electroencephalograms of all patients with hepatic failure exhibited the usual diffuse slow activity. During infusion of the 5 HTP all 3 showed a diminution of the slow activity and an increase of fast activity, representing a change in the record toward a more normal pattern. None of the patients developed a completely normal pattern, however. Fig. 1 is an example of the tracing from one patient in liver failure who had a very slow record before administration of the 5 HTP. The lower half presents the recording after infusion of 5 HTP (which took 20 minutes) was completed. With due allowance for the EKG artefact, it is obvious

that there is an increase in faster frequencies and a decrease in the slow activity. These changes lasted for about an hour and the record gradually reverted to its previous form. The patient expired about 8 hours later.

In the controls there was no change in the electroencephalogram of a patient with subdural hematoma on administration of 5 HTP, even though this record showed markedly slow activity bilaterally. In one patient with peptic ulcer and normal EEG, and in another patient with uremia and a mild diffusely slow EEG, the administration of 5 HTP produced no gross EEG changes.

All patients showed an increased excretion of 5 HIAA, amounting to about 80% of the expected amount of 5 HTP administered (calculated as the 1 form) within 6 hours. This is somewhat more rapid than the excretion noted on larger dosage(13). The fact that all patients did convert the 5 HTP to 5 HIAA equally well shows that the liver disease had no effect on conversion of 5 HTP. This furnishes further indirect evidence that the liver is concerned only with the hydroxylation of tryptophane to 5 HTP, since the 3 patients with liver failure also had low initial urinary excretion of 5 HIAA.

This evidence of a deficiency of an essential metabolite for brain caused by failure of the liver adds to the increasing understanding of the dependence of cerebral upon hepatic function. It also suggests that there might well be other essential precursors required by the brain, many of which might be supplied only by the liver. This would broaden the concept of the mechanism of hepatic coma to include substances which fail to appear in normal amounts in the circulation as well as those which accumulate excessively.

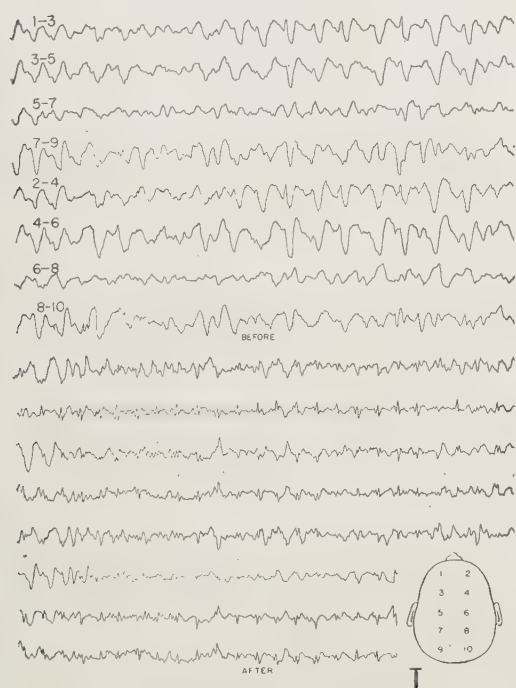


FIG. 1. Calibration 100 microvolts and 1 sec.

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Ovarian Weight and Responsiveness to Gonadotrophin Throughout the Estrous Cycle of Mice. (23268)

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Information about ovarian weight and capacity to respond to gonadotrophin at each stage of the estrous cycle is necessary for investigation of the effects of gonadotrophin on the ovary of the intact adult female. Astwood(1) reported the average uterine weight during estrous cycle of the rat, but data on weight of ovary during the cycle are not available for either rat or mouse. Folley and Malpress(2) stated that the stage of the estrous cycle had no effect on response of the cow ovary to exogenous gonadotrophin. Rowson (3) and Willett *et al.*(4) reported that cows gave a greater ovulatory response when gonadotrophin was administered during the follicular phase of the cycle. The purpose of this investigation was to determine if the weight of the mouse ovary and its capacity to respond to a constant level of equine serum gonadotrophin fluctuated during the estrous cycle.

Methods. Virgin female mice of the C57BL/6JAX strain, 90 to 120 days of age were used. To determine the stage of the estrous cycle, a vaginal smear was taken the day the mouse was put on experiment. The smears were air-dried and stained with 0.5% methylene blue in saline solution; the stage

of the cycle was designated according to the scheme of Allen(5). Routine daily vaginal smearing prior to use of animals was not done because this tended to upset the cycle in a high percentage of the mice. *Average ovarian and uterine weights during the estrous cycle.* For this phase of the investigation, 74 mice were chosen at random from the colony and vaginal smears were made. They were sacrificed and the right ovary and uterine horn excised, dissected free of fat, and weighed on a torsion balance. The average weight data obtained are summarized in Table I. With the exception of late metestrus (M_2) and proestrus (P), the average and median ovarian weights were similar throughout the cycle. The values for those 2 stages were not reliable because the number of mice was not sufficient. Uterine weights shown here are at variance with similar data that Astwood compiled for the rat(1). The rat data showed a peak uterine weight at proestrus, and a decline that began at estrus and continued until it reached its lowest ebb on the first day of diestrus. The failure to decline rapidly at estrus (O) and early metestrus (M_1) reported here may represent a fundamental difference in the ovarian hormone secretion pattern of rat *vs.* mouse. In a group of 60 mice from 5 to 11 months of age, the average uterine weights at

* Operated by Union Carbide Nuclear Co. for the U. S. Atomic Energy Commission.

TABLE I. Ovarian and Uterine Weights at Each Stage of the Estrous Cycle.

	O	M ₁	Stage of cycle		P	All
			M ₂	D		
No. of mice	14	21	3	33	3	74
Avg wt of right ovary (mg)	3.6	4.0	2.9	3.9	3.5	3.8
Median of right ovary wts	3.8	3.9		3.9		
Avg wt of right uterus (mg)	45.8	45.2	31.1	30.8	26.3	37.6

each stage of the cycle were almost identical to those shown here. The number of mice taken at estrus and early metestrus may seem high for a random sample, but it has been reported that the period of vaginal cornification persists as long as the period of diestrus in C57BL mice(6).

Results. Response to gonadotrophin injected at different stages of estrous cycle. A procedure was designed in which each animal was used as its own control to conserve mice and to attempt to overcome the variability encountered in mature mice. The animals were anesthetized with nembutal and the entire right half of the reproductive tract was exteriorized through a ventral midline incision; the right uterine horn was cut just above the cervix and the right reproductive tract was removed by blunt dissection of the mesenteries. The right ovary and uterine horn were trimmed free of fat and weighed separately on a torsion balance. The body wall was then sutured and the animal was given a subcutaneous injection of 20 i.u. of equine serum gonadotrophin[†] (PMS), contained in 0.25 ml of distilled water. Seventy-two hours later the animals were sacrificed and the left ovary and uterine horn removed and weighed. The differences in weight between the right and left sides were taken as the measure of the degree of stimulation.

Fifteen control mice were treated in the manner just outlined except that distilled water was substituted for the PMS solution. Seventy-two hours after removal of the right side of the reproductive tract, the average weight of the left ovaries had declined approximately 25%; the amount of decline could not be related to the stage of the es-

trous cycle during which the operation was performed.

Table II is a summary of ovarian and uterine weights before and after gonadotrophin administered during the estrous cycle. There appeared to be no difference in ovarian weight response to PMS during estrus, early metestrus, and diestrus. Too few mice were treated during proestrus and late metestrus to furnish a basis for any conclusions.

Many workers merely divide vaginal smears into two categories, one called "estrous smears" containing epithelial cells only (this would include O, M₁, and P by Allen's scheme); the other category usually called "diestrus" containing epithelial cells and polymorph leucocytes (Allen's M₂ and D). On the right side of Table II the results are tabulated by this scheme, and again there appeared to be no difference in the capacity of the ovaries of either group to respond to PMS.

The uterine weight before PMS varied with the stage of the cycle. However, the stage of the cycle had little if any effect on the average uterine weight after PMS.

Discussion. The average weight of the mouse ovary did not fluctuate during estrus, early metestrus, and diestrus; but the cyclic fluctuation of the uterus adequately demonstrated that ovarian hormone secretion was changing with the cycle. Thus, in the mouse, ovarian weight is not a valid quantitative criterion of secretion. It is possible that the weight of the mature follicles and their fluid are about the same as that of the corpora lutea, and in the mouse a shift from follicular to luteal phase would not be accompanied by any significant ovarian weight change.

The ovarian weight increase after gonadotrophic stimulation was about the same when the stimulus was begun in estrus, early metestrus, and diestrus. This indicated that the capacity of the ovary to respond to gonado-

[†] The equine serum gonadotrophin "Equinex" was supplied in generous amounts by Dr. John B. Jewell of Ayerst Laboratories, Inc.

TABLE II. Weight Responses of the Ovary and Uterus to 20 i.u. of PMS at Each Stage of the Estrous Cycle.

	Vaginal smear					Epithelial cells only	Epithelial cells and leucocytes	All stages
	O	M ₁	M ₂	D	P			
No. of mice	9	14	2	15	3	26	17	43
Avg wt of right ovary (mg)	3.6	3.8	3.2	3.6	3.5	3.7	3.5	3.6
" " " left " "	6.2	6.6	4.8	6.4	7.4	6.7	6.2	6.4
Increase of ovarian wt (%)	73	75	50	79	108	78	76	77
Avg wt of right uterine horn (mg)	46.0	44.9	36.7	31.0	26.3	43.1	35.9	38.9
" " " left " "	43.1	51.1	36.5	43.5	42.0	47.3	42.6	45.5

trophin is not influenced by the stage of the cycle and hence that ovarian responsiveness is not a factor involved in the cyclic phenomena. The similarity of uterine weights after stimulation at different stages of the cycle suggests that ovarian hormone secretion capacity is not influenced by the stage of the cycle either.

These data indicate that adult animals of the same age can be used to bioassay gonadotrophin by ovarian weight increase if great accuracy is not necessary. Furthermore, the stage of the cycle can be disregarded as a factor that influences the outcome of the assay.

Summary. 1) The average weight of the right ovary of C57BL/6JAX mice 90 to 120 days old was about the same in mice killed in estrus, early metestrus, and diestrus. The average uterine weight varied according to

the stage of the cycle. 2) The average ovarian weight increase after 20 i.u. of equine serum gonadotrophin was the same when the gonadotrophin was administered in estrus, early metestrus, and diestrus. Also the average uterine weight after ovarian stimulation was about the same, regardless of the stage of the cycle in which the stimulus was begun.

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Radioactivity in the Uteri of Rats Following Injection of Estrone-16-C¹⁴* (23269)

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The distribution and localization of gonadal steroids appear to be more closely related to their metabolism and excretion than to organs having sites of physiological action (1,2,3,4). In this report an attempt has been

made to define the nature of the radioactivity in the uteri of rats injected with estrone-16-C¹⁴.‡

Materials and methods. Adult ovariectomized rats (7 days) were injected subcutaneously with 5 µg of estrone-16-C¹⁴ (12,000 c.p.m.) daily for 3 days. Uterine luminal fluid was withdrawn from the distended uteri

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† Present address: Am. Cyanamid Co., Pearl River, N. Y.

‡ Charles E. Frosst and Co., Montreal, Canada.

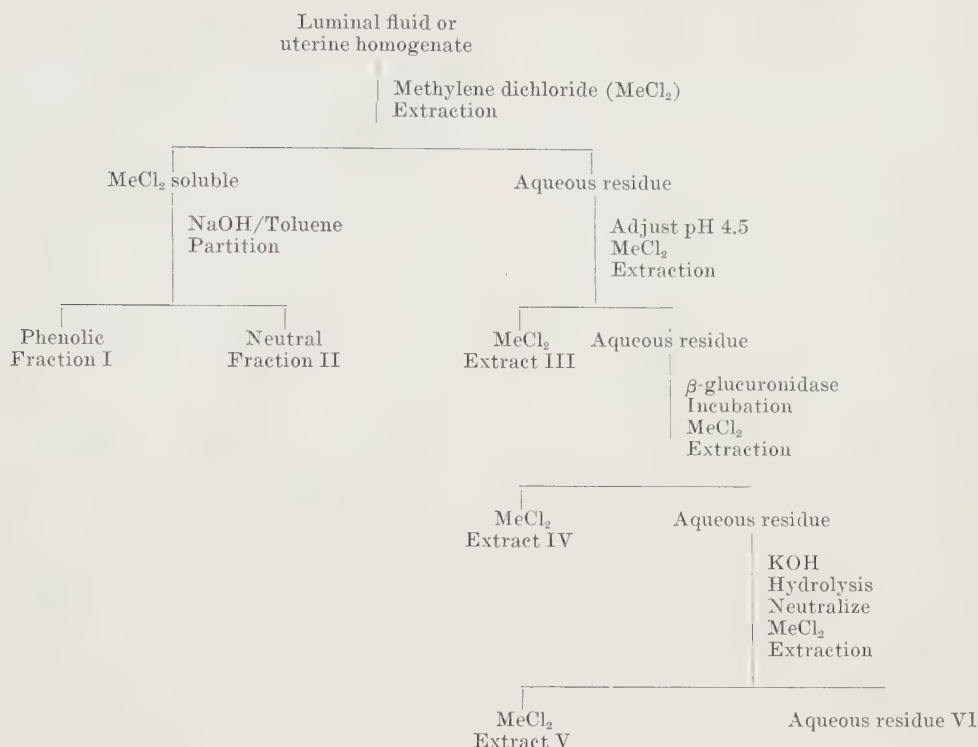


FIG. 1. Flow sheet for separation of radioactivity.

by syringe to avert contamination with blood. The uteri were then stripped of mesentery, washed in distilled water and immediately frozen on dry ice. The pooled samples of luminal fluid and uteri from 410 animals were 169 ml and 77.3 g, respectively. The uteri, in 500 ml distilled water, were homogenized in a Waring blender for 5 minutes. The extraction procedure for uterine fluid and the uterine homogenate is outlined in Fig. 1. Both uterine luminal fluid and uteri were extracted with dichloromethane (MeCl_2) and the extract partitioned between 1N NaOH and toluene. The aqueous residue was adjusted to pH 4.5 with glacial acetic acid and extracted with MeCl_2 . β -glucuronidase (200,000 units) was added to the acid residue and the mixture was incubated for 48 hours at 37°C. Following extraction with MeCl_2 , the aqueous residue was adjusted to 4N with KOH, hydrolyzed for 2 hours at 90°C, neutralized and extracted with MeCl_2 . All samples were counted in a windowless gas-flow counter for a period to insure $\pm 5\%$ accuracy,

and corrected for background radiation.

Results. A tabulation of radioactivity in rat uterine luminal fluid and uteri is summarized in Table I. The recovered radioactivity in the phenolic fractions from both luminal fluid and uteri (79 c.p.m.) is approximately .005% of the total injected radioactivity. Bioassay of the phenolic fraction by the technic of Astwood(5) proved negative. No steroidal radioactivity was present as either a glucuronide-conjugate or bound to protein. The only appreciable radioactivity was present in the aqueous residue. A similar

TABLE I. Distribution of Radioactivity in Uterine Luminal Fluid and Uteri.

Fraction	Radioactivity (c.p.m.)	
	Uterine luminal fluid	Uteri
I	19	60
II	0	0
III	0	0
IV	0	0
V	0	0
VI	210	36,000

radioactive component(s) in the aqueous phase has been noted in plasma and liver from estrone-16- C^{14} treated rats. These results indicate that the rat is capable of degrading the steroid nucleus at ring D. Heard, *et al.*(6) has reported the presence of radioactive CO_2 after administration of isotopic estrone, however, other workers have been unable to detect any respiratory $C^{14}O_2$ (2,7). A more definite identification of the radioactive compound(s) in the aqueous residue is now in progress.

The lack of any appreciable steroidal radioactivity or biological activity in either luminal fluid or uteri seems to indicate that once the steroid has produced its physiological effect in the uterus, it either leaves the uterus to enter the general circulation or is degraded.

Summary. The partitioning of radioactivity in uterine luminal fluid and uteri from rats injected with estrone-16- C^{14} is described. Though a minute amount of radioactivity is

present in the phenolic fraction, the major portion is present as a water soluble component. No steroidal radioactivity was present as either a conjugated or protein-bound form.

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Adaptation of Equine Abortion Virus to HeLa Cells.* (23270)

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Goodpasture(1) has presented histological evidence that equine abortion virus (EAV) is infectious for the human amnion. The agent has been propagated in tissues obtained from the natural host(2). This reference contains a suitable review of the literature. A search for readily susceptible cells to investigate further the nature of chemical changes induced in hamster liver cells infected with EAV(3) led to the use of HeLa cultures. In the present communication the propagation of the virus is reported.

Materials and methods. The tissue culture cells used have been adapted to horse serum in this laboratory for over 3 years. Stock cultures were maintained in a mixture of 40% horse serum, 2% chick embryo extract, and

58% Earle's balanced salt solution (BSS), and were subcultured approximately every 10 days. The virus strain used in this study deserves some comment. Preliminary experiments indicated that "native" virus from infected horse tissue could not be propagated in HeLa cells. Therefore, it seemed advisable to employ a strain modified to another host. The virus (strain F)(4) used to initiate this study had been adapted to hamster liver through 86 passages. Subsequently, the agent had been subjected to 2 alternate passages in young hamsters and HeLa cells maintained in ascitic fluid, followed by 12 successive passages in HeLa cells. Material from the last trial was reinoculated into hamsters. The infected liver used as inoculum was frozen and ground, diluted 1:10 in physiological saline and centrifuged for 5 minutes at 5,000 rpm.

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Prior to inoculation tissue cultures in 30 ml serum bottles were washed 3 times with BSS. Three or more cultures were inoculated with 0.2 ml of 1:10 virus suspension and allowed to stand at room temperature for 30 minutes. Control cultures received 0.2 ml of BSS. All cultures were incubated at 37°C with 2 ml of a 50-50 mixture of human ascitic fluid and BSS, which was changed every other day. The cultures were incubated from 3-7 days, harvested, ground and restored to volume with the last change of nutrient fluid. Fresh cultures were inoculated with 0.2 ml of the previous passage. For histological studies coverslips, 11 x 22 mm, were introduced into the culture vessels at the time of subculture. Suitable inoculated cultures and controls were fixed in Zenker-acetic acid and stained with H&E. *Complement-fixation methods.* The conventional method used in this study has been utilized elsewhere for identification of the agent(5,6). The origin and preparation of the various control sera and antigens have been previously documented(2,6). Hence, only the barest essentials will be mentioned. The tissue culture cells used as antigens, and all other reagents, were stored at -50°C. Immediately prior to use the cells were thawed, washed free of nutrient fluid, ground in physiological saline, and clarified by centrifugation at 2,500 rpm for 10 minutes. In the test proper 0.25 ml of a 1:10 dilution of antiserum was pipetted into tubes containing 2-fold 0.25 ml of serial dilutions of antigen and 2 full units of guinea pig complement in 0.5 ml. Following overnight fixation at 4°C, 0.5 ml of sensitized sheep red cells were added and the tubes incubated for ½ hour at 37°C. Adequate controls were included for each reagent.

Results. Serial propagation. In very early passages there was considerable variation in the gross evidence of infection. Some cultures appeared normal, others showed focal to general aggregation into masses of oval dark cells. This process of retraction and clumping formed spaces simulating plaques. Beginning with the 16th passage these cytopathogenic effects have been fairly constant. The incubation period gradually decreased from 7 to 3 days as the virus became more cyto-

TABLE I. Complement-Fixation Reactions.

Antigens		Serial dilution of virus	Antigen titration end-points
EAV original inoculum			128
Normal HeLa cells			0
8th passage tissue culture virus		10 ⁻⁸	8
27th passage	<i>Idem</i>	10 ⁻²⁷	8
35th passage	"	10 ⁻³⁵	16

pathogenic. When the culture showed general clumping of cells into necrotic masses which were readily detachable from the glass, the culture was terminated. The identification of intranuclear inclusions characteristic of the infection in susceptible species(2) is of some interest. Characteristic inclusions can be identified in 24 hour cultures stained at random including the 1st and 35th. Older cultures were unsatisfactory because cells would not adhere to the cover slips.

Identification of tissue culture virus. Hamster propagated EAV is lethal for 3-week-old hamsters with LD₅₀ titers varying from 10⁻⁶-10⁻⁸ and inclusions were demonstrated in practically all the parenchymal cells of the liver. HeLa cell cultures beginning with the 3rd and subsequently with every other passage have been pathogenic for young hamsters; the titer varying from passage to passage. Undiluted material from some cultures, in spite of pronounced cytopathogenic effects in tissue culture, will not kill all inoculated animals. A rare passage will yield a LD₅₀ titer of 10⁻⁴. Data from passages 3-35 apparently indicate no real decrease or increase in virulence of tissue culture virus for hamsters. The dilution of the original inoculum, as a result of serial passage, has exceeded 10⁻³⁵. If dilution due to change of nutrient fluid is taken into consideration, then the dilution exceeds 10⁻⁸⁰.

The cultured virus appears to be adequately identified by the complement fixation reaction. The data shown in Table I are adequate proof that the serological specificity of the agent has not been altered by passage in HeLa cells. The antigen titration end-point is defined as the highest dilution of antigen which results in complete fixation of complement with a 1:10 dilution of antiserum.

Summary. The adaptation of equine abortion virus to HeLa cells through 35 serial passages is reported. Characteristic intranuclear inclusions occur in cultures selected at random. The agent fixed complement in significant dilutions and remained pathogenic for hamsters.

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Influence of Stress on Distribution of Endotoxin in RES Determined by Fluorescein Antibody Technic.* (23271)

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The site and mode of primary action of endotoxin of Gram negative bacteria in animal tissue is unknown. In an attempt to elucidate this problem, results of parenteral injection of toxin have been studied under a variety of conditions. Among the many experiments, which have been performed, are studies of the effect of toxin after a single injection in normal animals and in animals treated with cortisone, thorotrast, x-irradiation and after two spaced injections(2,8,13,14). Since cortisone and thorotrast can substitute for the preparatory dose of toxin in the generalized Shwartzman reaction, it was considered of interest to determine how animals dispose of toxin under these conditions. Therefore, distribution and retention of the endotoxin of *Salmonella typhosa* after intravenous injection in normal animals and in animals stressed by the aforementioned agents was followed by the Coons' fluorescein technic.†

Materials and methods. The direct fluorescein tagging of gamma globulin according to the method of Coons and Kaplan(5) was employed. Normal and typhoid immune gam-

ma₂ globulin fractions were purified by the method of Nichol and Deutsch(11). The endotoxin of *Salmonella typhosa*, strain Edward, was purified by the method of Webster, *et al.* (15). All experiments were performed in American Dutch rabbits weighing approximately 1 kg. The deposition of toxin was studied in 15 normal rabbits after a single intravenous injection of 2 mg of toxin. The animals died or were killed between 20 minutes and 47 hours after injection. In the cortisone study, 15 rabbits were injected intramuscularly daily for 3-4 days with 25 mg of cortisone acetate.‡ After third injection of cortisone, 2 mg of toxin was injected intravenously. The animals died or were killed between 30 minutes and 46 hours after toxin injection. Fourteen animals were injected intravenously with 3 ml of thorotrast§/kg body weight. Six hours later, 2 mg of endotoxin was injected intravenously. The animals died or were killed between 3 hours and approximately 15 hours after toxin injection. Fourteen animals were subjected to 400 roentgen units, total body irradiation. Twenty-four hours later, 2 mg of toxin was injected intravenously. The animals died or were killed

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† The light source was an Osram mercury arc in a Reichert housing equipped with Schott BG 12 filter, 3-3½ mm thick, and a Wratten 2A and 15G filter. We are indebted to Dr. George Price for his assistance in selection of the optical system used.

‡ Cortisone acetate, aqueous suspension, Upjohn Co., Kalamazoo, Mich.

§ Thorotrast, 24-26% thorium dioxide by volume, Testagar and Co., Detroit, Mich.

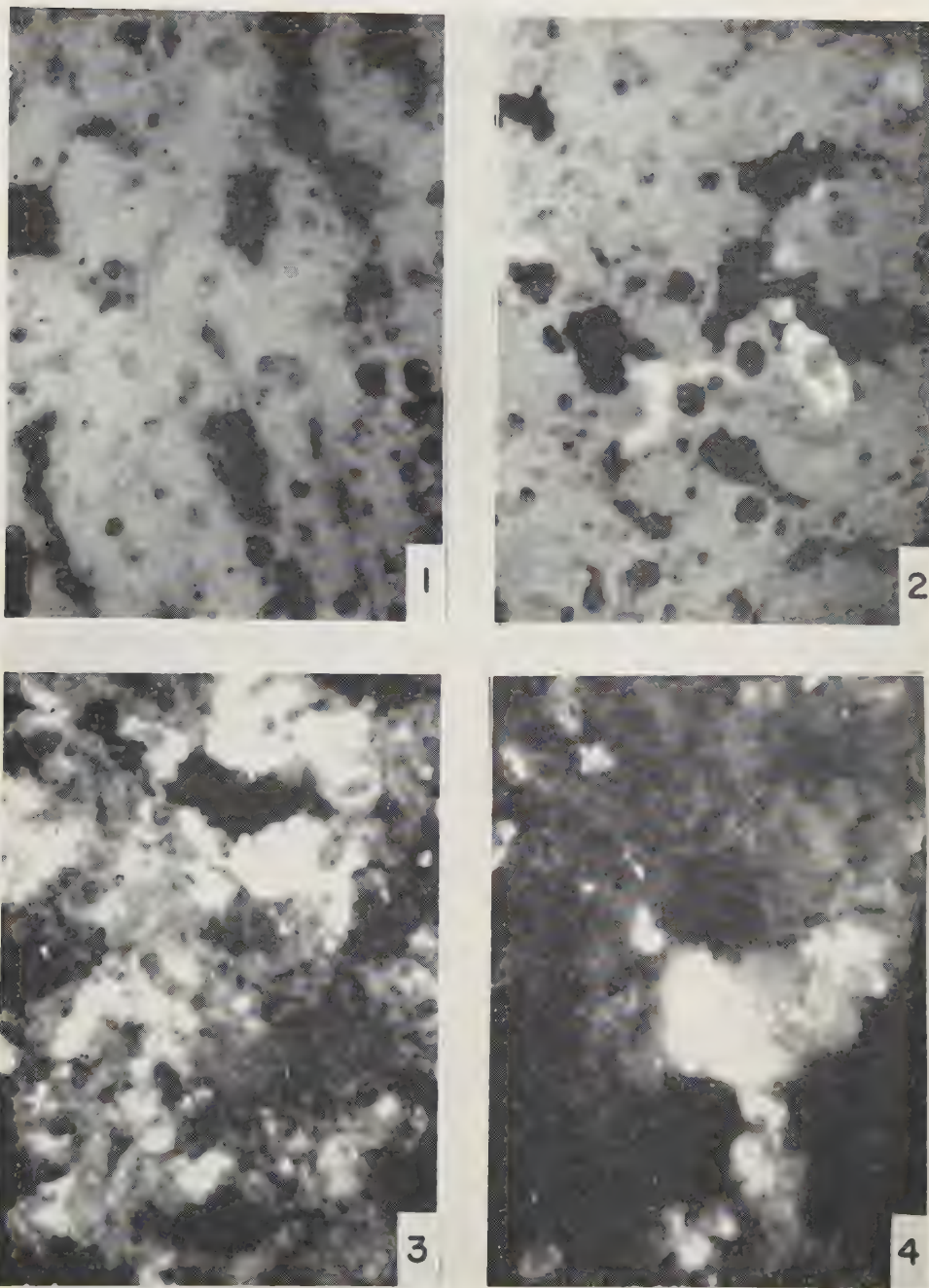


PLATE I. Photomicrographs demonstrating appearance of control and positive tissue sections. Bright areas indicate site of toxin-fluorescein tagged antibody complexes.

1. Duplicate control section of #2 showing inhibition of specific staining by first overlaying section with untagged immune sera and then staining with fluorescein tagged immune gamma globulin.
2. Section of liver stained with fluorescein tagged immune gamma globulin, 1+ reaction.
3. Section of spleen stained with fluorescein tagged immune gamma globulin, 3+ reaction.
4. Section of lung stained with fluorescein tagged immune gamma globulin, 3+ reaction.

between 30 minutes and 54 hours after injection. A series of animals were given an intravenous injection of 250 μg of toxin. Twenty-four hours later the 11 surviving animals were injected intravenously with 2 mg of toxin. The animals died or were killed between 30 minutes and 21 hours after second injection. Blocks of tissue from the liver, spleen, adrenal, lung, kidney and heart were frozen at -70°C and stored at -20°C . Frozen sections, measuring 2-4 μ in thickness, were subsequently cut and stained by the Coons' technic. Three types of controls were employed. Duplicate positive sections were negative when stained with conjugated normal gamma globulin. Specific positive staining was inhibited when the sections were first treated with specific immune, untagged serum. Sections of tissues of uninjected animals and animals which were pretreated with cortisone, thorotrast and x-irradiation, but which had received no toxin, were negative when stained with conjugated immune gamma globulin. The sections were examined microscopically and arbitrarily graded by the amount of toxin observed per high power field. Although it is not possible to obtain quantitative results with this technic, certain obvious and striking differences were observed between normal and stressed animals.

Results. The toxin, when present in any experiment, was found in Kupffer cells of the liver, in the red pulp of the spleen with only an occasional particle in the white pulp, in cells in and near the alveoli of the lung and within the lumen of vessels, in the glomeruli and connective tissue of the kidney, in the connective tissue and in cells lining the vessels of the heart, and in the lining cells of the adrenal sinusoids. In normal animals, the toxin was removed from the circulating blood quickly by the RES, reached a maximum within a short time and then began to decline. The reduction of toxin in the organs with time was most noticeable in the spleen and lungs, the liver maintaining a rather constant pattern over the period of the experiment. Toxin was either absent or minimal in adrenals, heart and kidneys.

In contrast to this, the level of toxin in cortisone treated animals remained high in

the spleen and lungs, as well as in the liver, during the period of the experiment. Initial phagocytosis was not curtailed but degradation and elimination of toxin was obviously reduced. An analogous situation occurred in the irradiated animals. Initial phagocytosis was not affected but there was retention of the toxin by the spleen, lung and liver. In both groups of animals the survival time was increased.

The opposite effect was noted in thorotrast treated animals. The RES, engorged with thorotrast particles which fluoresced orange, was able to remove little or none of the circulating toxin. This was reflected in an increased death rate. An impairment of phagocytosis by the RES was also observed in animals given an initial, smaller injection of toxin. The effect was most noticeable in spleen and liver sections. This curtailment of phagocytosis was again reflected in an enhanced death response. However, whereas in the thorotrast experiment, the effect was due to a blockading of the RES by thorium particles, in this experiment, it did not appear to be a blockading effect, since only minimal amounts of toxin were observed in the RES after the first injection.

With an occasional exception, the heart, adrenals and kidneys of all animals showed only a minimal amount of toxin. Nine animals exhibited bilateral cortical necrosis of the kidney, three each from the groups treated with cortisone, x-irradiation and a preliminary injection of toxin. Gross changes in the kidney were not correlated with large accumulation of toxin in the organ.

Discussion. Studies on distribution in animals of endotoxin labelled with radioactive isotopes have been reported (1,3,12). The findings of this study, employing a technic dependent upon immunologic specificity, are essentially in agreement with these earlier reports.

There is a certain analogy between the ability of cortisone, thorotrast, and an initial injection of toxin to prepare for the Schwartzman reaction and the results in this study. Thorotrast and the first injection of toxin by incapacitating the initial phagocytic ability of the RES, probably keeps the provoking dose

TABLE I. Distribution of Endotoxin with Time in Normal and Stressed Animals.

No. of animals	Treatment	Death	Died/killed	Liver	Spleen	Lung	Heart	Adrenal	Kidney
5	None	20 min.- 5 hr	3/2	1-2+	1-2+	2+	±	±	±
10	"	10 hr -47 hr	4/6	1+	+	+	±	±	±
3	Cortisone	30 min.- 2 hr	2/1	1+	3+	2+	±	+	±
12	"	22 hr -46 hr	1/11	1+	2-3+	1+	±	±	±
8	X-irradiation	30 min.- 5 hr	4/4	1-2+	2+	1+	±	±	±
6	"	8 hr -54 hr	0/6	1+	2+	1+	±	+	±
8	Thorotrast	3 hr - 5 hr	4/4	±	±*	+	±	±	±
6	"	6 hr -15 hr	6/0	+	±*	+	±	±	±
9	2 spaced inj. of toxin	30 min.- 5 hr	9/0	+	+	1+	±	±	±
2		7 hr -21 hr	1/1	1+	1-2+	1+	±	±	±

* Majority were negative.

of toxin in the systemic circulation where it can effect its deleterious action at its primary site for a longer period of time. Cortisone, while it does not affect initial phagocytosis, curtails normal degradation and elimination of toxin thus providing a potential, constant source of reinoculation. With normal cell breakdown, the toxin is released and enters the circulation, free once again to act at its primary site. That the toxin remains antigenically unchanged during its sojourn in the RES is evidenced by the fact that the fluorescein tagged antibody specifically complexes with it during this period. This interruption of normal metabolism of toxin by the RES was found also in irradiated animals. Similar observations regarding digestive curtailment of the RES in cortisone treated and irradiated animals are reported by other investigators(4,6,7,9,10). The reinfections, continuing bacteremia, activation of quiescent infections in cortisone and x-rayed animals could be explained on this basis.

Summary. 1. Deposition of endotoxin of *Salmonella typhosa* was followed in normal and stressed rabbits by the fluorescein tagging technic. 2. Pretreatment with cortisone and x-irradiation did not affect initial phagocytosis of toxin but inhibited degradation and elimination of toxin by the RES. 3. Pretreatment with thorotrast and preliminary injection of toxin caused depression of initial phagocytic functioning of the RES to a subsequent

injection of toxin. 4. Our findings in their possible relation to the generalized Shwartzman reaction were discussed.

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Hemosiderin. Isolation from Horse Spleen and Characterization.*† (23272)

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Behrens and Asher(1) separated hemosiderin from horse spleens by utilizing fractional sedimentation with the aid of organic liquids of high specific gravities and analyzed this material for iron, phosphorus, calcium and nitrogen. Recently, Greenberg(2) described a procedure for separating hemosiderin by means of differential specific gravity centrifugation in a concentrated aqueous potassium iodide solution.

The present investigation is concerned with (1) fractional sedimentation of a water insoluble, iron rich complex (hemosiderin) from horse spleen by using salt solutions of relatively high specific gravities and (2) an analysis of some of the constituents of this material.

Methods. Isolation of Hemosiderin. Frozen horse spleens were thawed at 4°C. The tissues were cut into small pieces and 80 g portions were minced to fine suspension in 400 ml of cold 0.9% NaCl in a Waring blender run at slow speed. The suspensions from 1000 g of spleen were pooled, filtered through a double layer of gauze, the filtrate was centrifuged and the supernatant solution discarded. The residue was extracted repeatedly with 1500 ml of 1 M NaCl to remove the nucleoprotein, detected as a precipitate after diluting the extract with water to yield a 1% saline solution. This step was essential for obtaining a final product free of gummy material. The extracted residue was stirred in 600 ml of saturated KBr solution (sp. gr. 1.38) and the mixture centrifuged. If a considerable amount of material floated on the surface of the supernatant solution, it was re-suspended in KBr solution and recentrifuged. The residues were sedimented successively in a 4.42 M KI solution (sp. gr. 1.52) and in a saturated KI solution (sp. gr. 1.72). The

hemosiderin was treated with distilled water until the washings were iodide free. The material was dried by lyophilization and stored over P_2O_5 . *Analytical procedures.* *Iron.* The analysis was done by iodometric procedure(3). About 12 mg of hemosiderin were ashed in platinum boat with H_2SO_4 plus HNO_3 (4). The ash was dissolved in 25 ml of 1 N HCl, the solution evaporated over open flame and finally brought to dryness on a steam bath. The residue was dissolved in 50 ml of 0.3 N HCl, one drop of bromine was added to convert the iron to the ferric form and the excess bromine was removed by boiling until the final volume was about 25 ml. Three grams of KI were dissolved in this solution, allowed to react with iron in the dark for 15 minutes, and the liberated iodine was titrated with 0.02 N sodium thiosulfate. *Phosphorus.* The ash obtained by above method was analyzed by the Fiske-Subbarow method(5). Nitrogen was determined by the micro-Dumas method.

Results. The weights of 9 spleens ranged from 550 to 1300 g and the yield of hemosiderin varied from a trace to 3 g/spleen. It was not possible to predict the yield of hemosiderin by gross inspection of spleens. Data for iron, nitrogen, phosphorus and ash concentrations of hemosiderins prepared from 9 individual horse spleens are shown in Table I. Variations in concentrations of these constituents indicate that hemosiderin does not have a constant composition. Iron concentrations vary from 24 to 36% which represent a range of 47 to 68% of the hemosiderins when expressed as $Fe(OH)_3$. An inverse relationship exists between iron and phosphorus and between iron and nitrogen; a direct relationship is observed between iron and ash concentrations.

In characterizing hemosiderin, Greenberg (2) calculated μg nitrogen/mg iron and obtained values of 320 and 290 for 2 preparations. In Table I the values for this ratio

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TABLE I. Iron, Ash, Nitrogen and Phosphorus Concentrations in Hemosiderin.

Sample	Iron	Ash	(%) Nitrogen	Phosphorus	$\frac{\mu\text{g N}}{\text{mg Fe}}$
1	30.7, 31.7				
2	29.5, 29.6	47.5, 47.7			
3	24.5, 25.0			3.05, 3.28	
4	28.0, 28.1		5.40, 5.40	3.04, 3.05	192
5	35.7, 35.9		3.70, 3.90	1.58, 1.66	106
6	25.0, 26.4	49.2, 49.5	5.43	3.44, 3.74	211
7	31.0, 31.8	50.3, 50.8	4.51, 4.81	2.38, 2.61	148
8	34.5, 36.4		4.15, 4.30	1.62, 1.71	119
9	24.7, 25.8	44.0, 45.0			

range from 106 to 211 for 5 different preparations. Similar calculations applied to hemosiderin analyzed by Behrens and Asher gave values of 111, 179 and 196 for samples containing 35.9, 31.6 and 29.3% iron, respectively.

To test whether each of 3 samples of hemosiderin had a constant composition, they were fractionated in organic liquids(1) with specific gravities greater than that of saturated KI. According to the data in Table II, the fraction which sediments at sp. gr. 2.18 has an iron concentration of about 41% in contrast to the original iron value of 36%. It can be seen that the iron values for the succeeding fractions decrease at lower specific gravities. These results would indicate that hemosiderin is an inhomogeneous mixture with different iron contents.

After slowly agitating 50 mg of hemosiderin in 5 ml of water at room temperature in a sealed test tube for 24 hours, the supernatant solution had a faint yellow tinge and yielded

TABLE II. Fractional Sedimentation of Hemosiderin by the Behrens-Asher Procedure(1). Hemosiderin was ground to fine powder in the Fisher Mortar Grinder.

	% iron in samples		
	2	5	9
Original	29.5, 29.6	35.7, 35.9	24.7, 25.8
Fraction with			
sp. gr. of: 2.18	No sed.	41.25, 41.60	No sed.
1.98	32.8, 33.1	36.1, 36.8	Traces
1.87	30.5, 30.9	32.6, 33.6	"
1.80	28.5, 28.5	No sed.	27.0, 27.4
<1.80	25.4, 25.8	" "	24.1, 25.0
Sp. gravity of 2.18: Ethylenebromide ($\text{C}_2\text{H}_4\text{Br}_2$)			
1.98: Mixture of $\text{C}_2\text{H}_4\text{Br}_2$ - CCl_4	(2:1)		
1.87: " "	" "		(2:2)
1.80: " "	" "		(2:3)

no precipitate with 10% trichloroacetic acid. The test for iron with $\text{K}_4\text{Fe}(\text{CN})_6$ was negative but on standing a blue precipitate developed, indicating that the yellow color was probably due to a small amount of colloidal iron hydroxide. In contrast, Behrens and Asher(1) and Behrens and Taubert(6) found that appreciable amounts of protein and iron were extracted from their hemosiderin samples, which indicates that hemosiderins prepared by different procedures yield different products.

Carbohydrates. After observing that hemosiderin gave a weak Molisch test, a hydrolysate of hemosiderin was routinely prepared by heating 100 mg of hemosiderin in 16 ml of 2 N H_2SO_4 in sealed tubes which were rotated slowly in boiling water for 4 hours. The hydrolysate was diluted to 64 ml with water and filtered. The filtrate was de-ionized by passage through a 13.2 x 2 cm column containing Dowex 50 (H^+ form), then through a 14.5 x 2 cm column containing Dowex-1-formate.[†] The de-ionized solution and column washings were dried by lyophilization.

The paper chromatogram for sugars present in the hemosiderin hydrolysate (Fig. 1) shows presence of galactose, mannose and fucose. According to the intensity of the color, concentrations of mannose and galactose are greater than that of fucose.

The (primary) cysteine-sulfuric acid reaction (PCyRI) for hexoses(8) was positive

[†] Dowex-1 anion exchange resin, 200 to 400 mesh, was washed with 1 N HCl and was converted to the formate by treatment with 0.3 M ammonium formate in 2 N formic acid until the chloride was completely displaced. Excess formate was removed by washing with water.

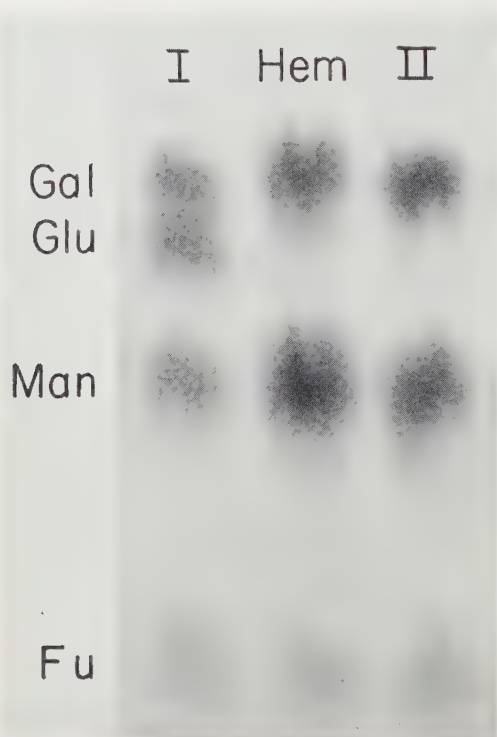


FIG. 1. Paper chromatograms of a hemosiderin hydrolysate (Hem) and of reference sugar mixtures (I and II). 100 mg hemosiderin were hydrolyzed and the deionized solution was lyophilized. Residue was dissolved in 50 μ of water and 20 μ l aliquot was applied to Whatman No. 1 paper. The reference solutions contained (I) galactose (Gal) 60 μ g, glucose (Glu) 60 μ g, mannose (Man) 60 μ g, and fucose (Fu) 30 μ g; and (II) galactose 90 μ g, mannose 120 μ g, and fucose 30 μ g in 20 μ l of water. These sugars were chromatographed with a butanol-water-ethanol (10:2:1) mixture for 3 days and developed by spraying with aniline hydrogen phthalate(7).

and the results with different samples of hemosiderin indicated that the concentration of these sugars was approximately 1%. By determining the values for D_{396} - D_{427} , it was shown that fucose was present at a concentration of about 0.1%.

The Dische-Borenfreund indole reaction (9) and the Elson-Morgan test as described by Boas(10), performed on neutralized hydrolysates, indicated that hemosiderin contained about 1% hexose amine.

A positive Lieberman-Burchard test for cholesterol was obtained in a carbon tetrachloride extract of hemosiderin and several amino acids were demonstrable by paper chro-

matography in hemosiderin hydrolysates. Histochemical analyses of hemosiderin deposited in tissue cells have also demonstrated the presence of polysaccharides, lipids and proteins(11,12).

Discussion. The earlier attempts(13,14) to isolate hemosiderin and to characterize it chemically were unsuccessful. Satisfactory preparations of hemosiderin(1) were first obtained by fractional sedimentation of dried, powdered horse spleen in organic liquids. A comparison of the analytical data, obtained in the present investigation, with those reported for hemosiderin by Behrens and Asher(1) shows that the nitrogen, iron, ash and nitrogen/iron ratios are similar but phosphorus concentrations and amounts of soluble protein and colloidal iron compounds are different. Greenberg(2) found much higher nitrogen/iron values for hemosiderin prepared by his method and also reported the presence of significant amounts of ferritin. His observation that apoferritin-cadmium crystals could be prepared from hemosiderin was confirmed.

The combination of hexose amine, galactose, mannose and fucose observed in hemosiderin has also been found in plasma γ -globulin and in myeloma proteins(15,16). In addition, fucose has been found to be present in blood group substances(17) and in red cell stroma(18).

Schwietzer(19) used x-ray diffraction to characterize hemosiderin obtained from horse spleens(1) and concluded that this material may contain a crystallized form of iron oxide identical with that of the minerals limonite or lepidocrocite (rubin glimmer). Six samples of hemosiderin, prepared in this laboratory, did not show a crystalline structure by the x-ray diffraction method.†

The data in the present investigation corroborate the earlier work of Behrens and Asher(1) who showed that the insoluble iron-rich particles, obtained from horse spleen, represent a number of hemosiderins with varying iron concentrations. In view of these find-

† The author is grateful to Dr. R. S. Mitchell of the Dept. of Geology, University of Virginia, for making these determinations.

ings, it would be appropriate to designate these materials as hemosidera.

Summary. 1. A method is outlined for isolating hemosiderin from horse spleen by sedimentation from salt solutions of different specific gravities. 2. The iron, nitrogen, phosphorus and ash concentration varied considerably in hemosiderin samples obtained from different spleens. Fractionation of these preparations by sedimentation in organic liquids showed that fractions could be obtained which varied in their iron concentrations from about 25 to 41% iron. 3. Hemosiderin has been found to contain hexoseamine, galactose, mannose and fucose.

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Fractionation of Radioactive B₁₂-Complex In Kidney Homogenates.* (23273)

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Rosenblum, *et al.*(1), demonstrated that among the various target organs of rats injected with radioactive B₁₂ (referred to hereafter as B₁₂*) kidneys contained initially the greatest amount of radioactivity per gram of the wet organ. However, a major portion of its radioactivity disappeared within 21 days, Harte, *et al.*(2). In contrast, the amount of radioactivity initially retained by the liver and pancreas of these animals decreased only little even after as long as 90 days after injection.

Furthermore, administration of non-radioactive B₁₂ to rats previously injected with B₁₂* resulted in a decrease in radioactivity in liver and pancreas but an increase in kidneys. These unique properties of kidney prompted us to study the manner in which B₁₂ is retained by this organ. To this end, the ability of the vitamin to combine with components of kidney tissue has been examined by various chemical fractionation procedures and differential centrifugation. The results of such studies are reported in this communication.

Methods. Preparation of Homogenates. Adult female rats from our stock colony were injected subcutaneously with 1.0 µg Co⁶⁰-

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TABLE I. Equilibrium Dialysis of Kidney Homogenates.

	—Radioactivity in cpm—			% bound
	Inside	Outside	Bound	
Homogenate from rat:				
1	507	142	365	56
2	485	112	373	63
3	436	94	343	64
B_{12} alone	244	224	20	

All kidneys were homogenized with 10 times their wt of distilled water. Five ml of these homogenates were used for each exp.

labeled B_{12}^* with a specific activity of 40 $\mu\text{C}/\text{mg}$. Forty-eight hours later, the animals were sacrificed and their kidneys were removed, trimmed of fat, and homogenized in a Potter Elvehjem Homogenizer with 10 times their weight of cold distilled water. *Analysis of Radioactivity.* Unless otherwise stated all samples for radiometric measurements were prepared by wet ashing with a mixture of nitric and sulfuric acid. To each sample was added 1 mg of cobaltous chloride which served as a carrier. After evaporation of the excess acids, the residue was transferred quantitatively to a planchet and dried. The radioactivity was then measured with a thin window Geiger Mueller counter.

Results. Determination of Bound B_{12} . (A) *By equilibrium dialysis.* Five ml aliquots of homogenates from kidneys of 3 rats were dialyzed with continuous shaking at 4°C for 48 hours against an equal volume of distilled water. As a control, 5 ml of a solution with a known amount of radioactive B_{12} was dialyzed simultaneously in a like manner. After 48 hours of equilibration, the fluids inside and outside the cellophane bags were analyzed for radioactivity. The results of a typical experiment (Table I) established satisfactory equilibration since radioactivity on either side of the bag containing the control sample was essentially the same. On the other hand, the inside fluids with the homogenate contained a greater amount of radioactivity than the corresponding outside fluids. The difference between these values may be considered as B_{12}^* bound to kidney tissues and is approximately two-thirds of the total radioactivity. The total radioactivity in the undialyzed kidney homogenates which dif-

fered somewhat with various rats, was also measured. Recovery of radioactivity in the fluids inside and outside the membrane was approximately 90% or greater.

(B) *By exhaustive dialysis.* Another means of measuring the bound B_{12}^* in the homogenate is to remove completely the free B_{12}^* by prolonged dialysis. Thus, 5 ml of the pooled kidney homogenate from several rats were dialyzed with gentle shaking against an equal volume of distilled water in a cold room with frequent changes of water. However, approximately 72 hours afterwards the dialysis bag was allowed to equilibrate with an equal volume of distilled water for an additional 48 hours. This was done to show that main bulk of the dialyzable B_{12} has been removed. Our findings showed that total radioactivity in the original homogenate was 123 μg but was reduced to approximately one-half (67 μg). The unbound B_{12}^* was almost completely removed since the total radioactivity in the last dialysate amounted to only 6 μg . The union between the vitamin and kidney tissue thus is not readily dissociated by dialysis.

(C) *Extraction with butanol.* Two other procedures were employed to determine the amount of the bound B_{12}^* in kidney tissue. The first was an attempt to extract the free vitamin in the kidney homogenate with butanol. Thus, 2 ml of a solution containing 50 μg of unlabeled B_{12} and saturated with ammonium sulphate was added to 2 ml of the kidney homogenate. This mixture was then extracted 5 times with 8 ml of butanol saturated with water. The radioactivity in the combined butanol extracts was measured after evaporation to dryness, and found to be 19 μg or 33% of the radioactivity in the whole homogenate (57 μg).

(D) *Precipitation of B_{12}^* -protein complex with acetone.* To 2 ml of the kidney homogenate was added 10 ml of redistilled acetone. After centrifugation radioactivity in the precipitate and the supernatant fluid was measured, and found to contain 35 μg and 20 μg respectively. All 4 independent methods demonstrate that two-thirds of the total radioactivity in the kidney was bound.

Differential Centrifugation. Differential

TABLE II. Distribution of Radioactivity among Various Cellular Fractions.

	Groups	Avg total cpm/pair of kidneys	% of whole homogenate			
			Nuclear (N)	Mitochon- dria (M)	Soluble (S)	Recovery
Exp. A	1	3404 ± 160*	39 ± 1	12 ± 1	40 ± 1	92
	2	1200 ± 131	39 ± 1	15 ± 1	38 ± 1	92
B	3 (Control)	1714 ± 168	38 ± 4	12 ± 1	44 ± 1	94
	4 (Post-inj.)	1998 ± 140	42 ± 1	9 ± 1	41 ± 3	92
	5 (Pre-inj.)	2860 ± 108	42 ± 1	10 ± 1	39 ± 3	91

* Stand. error of mean.

centrifugation is useful in the separation of particulates. It is of interest to determine distribution of the injected B₁₂* in the various fractions of kidney homogenates. In the first experiment 5 adult female rats were injected subcutaneously with 1 µg of B₁₂* administered in 4 equal doses during a 48-hour period. Forty-eight hours after the last injection the animals were sacrificed. The kidneys of each animal were trimmed of fat and homogenized with 10 times its weight of cold 30% sucrose solution employing a Potter Elvehjem Homogenizer. The homogenate was centrifuged at 2°C for 10 minutes at 800 g. The precipitate (nuclear fraction) was resuspended and recentrifuged twice in a 10 ml of cold 30% sucrose solution. The washings were combined and added to the original supernatant fluid and centrifuged for 30 minutes at 25,000 g. This precipitate is referred to as the mitochondrial fraction and the supernatant fluid as the soluble fraction. All isolated fractions as well as 2 aliquots of the original homogenate were assayed for radioactivity as previously described. The results demonstrate that approximately 38 ± 4% of the total radioactivity of the whole homogenate is found in the nuclear fractions, 12 ± 1% in the mitochondrial fraction and 44 ± 2% in the soluble fraction. Since in contrast to other target organs the kidney of rats loses the major portion of radioactivity within 3 weeks after parenteral administration of B₁₂*, it was of interest to ascertain whether loss of radioactivity from any one of these isolated fractions was responsible for this phenomenon. Eight adult female rats were injected with 2 µg of B₁₂* as previously described. Four of the animals were sacrificed 2 days (Group 1) and the remaining rats 16 days

(Group 2) following the last injection. The kidneys of all animals were fractionated by the differential centrifugation method. Microscopic examination showed the presences of only a few intact cells and some mitochondria in the nuclear fraction. The supernatant fraction contained no mitochondria and the mitochondrial fraction was found to be free from microsomes by dark field microscopy. These results (Table II Exp. A) demonstrate that in spite of a marked loss of radioactivity 16 days following administration of B₁₂*, distribution of radioactivity among the various fractions remains unchanged. This finding led to an examination of distribution of radioactivity in kidney homogenates under other experimental conditions known to affect amount of radioactivity of kidney homogenates. Fifteen adult female rats were employed in this experiment. Five animals were injected with 50 µg of unlabeled B₁₂ per day for one week preceding administration of one µg of B₁₂* to all animals. Forty-eight hours following the last injection of B₁₂* the 5 treated animals (pre-injection Group 5) as well as 5 additional ones (control Group 3) were sacrificed. The remaining rats (post-injection Group 4) were then injected with 50 µg of unlabeled vitamin B₁₂ per day for one week. Twenty-four hours following the last injection these animals were sacrificed. The kidney homogenates of all animals were fractionated immediately following sacrifice. The results (Table II Exp. B) again demonstrate that distribution of radioactivity among the various isolated fractions is the same regardless of total amount of radioactivity or experimental conditions imposed upon the animals.

Discussion. Numerous reports (Ross(3),

Rosenthal(4) and Pitney(5)), have demonstrated that serum contains mostly bound and a little free vit. B₁₂. More recently it has been shown that the capacity of serum to bind this vitamin may vary according to diseases, (Beard(6) and Davis(7)). The ability of an organ to perform certain functions may be decided by the amount of vit. B₁₂ retained by the organ. Therefore, it seems likely that information of the capacity of various fractions of tissue to combine with vit. B₁₂ is important. Although only radioactivity of Co⁶⁰ is measured in this study, we believe this measurement represents vitamin B₁₂, *per se*, since it has been shown that the vit. B₁₂, given by injection, is not altered in the body and is excreted in the urine in its original form(1,9). Our results indicate that approximately two-thirds of the radioactivity found in the kidneys of rats following parenteral administration of B₁₂* is bound to tissue components. Furthermore, the union between the vitamin and kidney tissue is not readily dissociated by dialysis, nor can the radioactive B₁₂ bound to kidneys be dissociated by subsequent injection of unlabeled B₁₂. The firmness in the binding is further demonstrated by our inability to remove, *in vitro*, the bound radioactive B₁₂ in kidney homogenate after the addition of a massive amount of unlabeled B₁₂ either by dialysis or extraction with butanol. The results obtained by the fractionation of kidney tissue by differential centrifugation indicate a relatively high concentration of vit. B₁₂ in the nuclear and soluble fractions and a low concentration in the mitochondrial fraction. It is of interest to point out that these findings differ from those of Swenseid, Bethell and Ackerman, who employed(8) a microbial assay for the

vitamin, demonstrated a high concentration in the mitochondrial fraction of mouse liver. This difference in results may be due to the different technics used, or to the innate characteristic of the organs or to the difference in the species of animals. Finally distribution of radioactivity among the various isolated fractions was found to be the same regardless of total amount of radioactivity or experimental conditions imposed upon the animals.

Summary. The amount of radioactive B₁₂ bound by substances in kidneys of rats pre-injected with this radiovitamin was found to be approximately one-half to two-thirds of the total radioactivity by 4 independent procedures. The amounts of radioactivity in the 3 fractions obtained by differential centrifugation were also determined with only a small portion in the mitochondria. Relative percentages of the radioactivity in these 3 components remained essentially unchanged as a result of pre-injection of unlabeled B₁₂.

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Effect of Hydrogenated Triolein on Utilization of Essential Fatty Acids in the Rat.* (23274)

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It has been reported(1) that the isomerization which occurs during the catalytic hydrogenation of linoleic acid and methyl linoleate produces cis and trans 9, 10, 11 and 12 mono-unsaturated fatty acids. It is therefore not surprising that trans fatty acid isomers have been found to occur in margarines and shortenings(2,3), since in their production, oils containing large amounts of unsaturated fatty acids—and a large proportion of these unsaturated fatty acids are the essential fatty acids, linoleic and linolenic—are either selectively or non-selectively hydrogenated. The nutritional fate of the trans fatty acids in hydrogenated fat has been the subject of several reports. Elaidic acid, trans 9, 10 octadecenoic acid, which is the geometric isomer of oleic acid, is not changed to oleic acid in the animal body. However, Barbour(4) reported that "isooleic acid" (a mixture of elaidic acid, Δ -12, 13 octadecenoic acid, and other isomers of oleic acid formed during the hydrogenation process), found in hydrogenated cottonseed oil, is deposited in the adipose tissues of the rat and is eventually utilized by the animal body as efficiently and effectively as other dietary fatty acids. Sinclair(5) used elaidic acid and trielaidin in studies of intermediary fat metabolism and reported that these compounds behaved exactly as did the normal fat components. Kohl(6) also reported on efficient utilization of elaidic acid

laid down in adipose tissues and phospholipids. In a study of the positional and stereoisomers of the unsaturated fatty acids produced on hydrogenation of vegetable oils, Melnick and Deuel(7) found that these isomers are not antimetabolites for natural oleic acid, but are used as nutrients. Microbiological methods were used in the studies concerned with the oleic acid isomers, and biological studies with the polyunsaturated fatty acid materials. Recently, Holman and Aaes-Jorgensen(8) reported that trans fatty acid isomers of linoleic acid fed to rats on an essential fatty acid deficient diet, containing cholesterol and hydrogenated coconut oil, did not induce recovery of the severe testicular degeneration which occurs in essential fatty acid deficiency. These trans isomers under the test conditions selected also worsened the skin condition and were found to be deposited in the animal fat. In experiments conducted in this laboratory directed toward the study of possible essential fatty acid antagonists, the presence of hydrogenated coconut oil has been shown to shorten considerably the time of depletion of essential fatty acids in the rat(9). In other long-term experiments(10) in which margarine oil, containing 35.3% trans fatty acid isomers, was fed in varying levels to rats for long periods of time, no interference attributable to this large concentration of trans fatty acids was observed when the criteria of growth, reproduction, lactation, survival, plasma and liver cholesterol levels and tissue pathology were studied. There was the possibility that the concomitant presence of adequate amounts of linoleic acid in this fat masks or counteracts any anti-metabolite action which may reside in the trans fatty acids contained in the margarine oil; the average ratio of trans to biologically active linoleic acid content in the test oils was 7:1. With this in mind, a partially hydrogenated triolein, containing no linoleic

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† Deceased April 17, 1956.

acid whatsoever but with a trans fatty acid content of 32.8% was prepared for use in feeding studies with rats. During this hydrogenation of oleic acid, migration of the double bond takes place so that positional cis and trans isomers also occur(11). The physical constants of this triglyceride are listed in Table I.

Methods. Male rats of the University of Southern California strain were placed at weaning on a diet adequate in all respects but deficient in fat. The diet contained 20% casein, 72% sucrose, 4% cellulose, 4% salt mixture and all required vitamins in adequate amounts.† The weight of the animals was recorded weekly. Animals were judged to be deficient in essential fatty acids when a plateau in weight and the appearance of typical skin symptoms (*i.e.* scaliness of tail and paws, rough coat and general unthrifty appearance) were observed, which usually occurred in from 12 to 16 weeks. After 16 weeks on the fat-free diet, the animals were divided into the following groups and placed in individual cages. Each animal received daily the supplement listed:

Group I	Control—continued on fat-free diet, no supplement
II	Fat-free diet + 250 mg hydrogenated triolein
III	Fat-free diet + 500 mg hydrogenated triolein
IV	Fat-free diet + 20 mg linoleate
V	Fat-free diet + 250 mg hydrogenated triolein + 20 mg linoleate
VI	Fat-free diet + 500 mg hydrogenated triolein + 20 mg linoleate
VII	Fat-free diet + 50 mg linoleate
VIII	Fat-free diet + 250 mg hydrogenated triolein + 50 mg linoleate
IX	Fat-free diet + 500 mg hydrogenated triolein + 50 mg linoleate

The animals were dosed for a period of 8 weeks during which time they were weighed

† A vitamin mixture containing 38.57% p-amino benzoic acid, 31.88% inositol, 12.75% ascorbic acid, 4.59% thiamine hydrochloride, 3.82% niacin, 3.82% Ca pantothenate, 1.72% riboflavin, 1.72% pyridoxine, 0.64% folic acid, 0.32% menadione, 0.16% biotin and 0.00004% B₁₂ was added to the diet at a level of 0.19%. A Nopsol solution containing 100,000 I.U. of vit. A/g and 20,000 I.U. of D/g was included at a level of 0.012%. 0.012% of α -tocopherol was added as well.

TABLE I. Chemical and Physical Properties of Hydrogenated Triolein.

Wiley melting point	97.7°F
Iodine value (Wijs)	67.2
Saponification value	195.8
Free fatty acids (%)	<0.05 as oleic acid
Fatty acid composition (%)	
Saturated (stearic)	20.9
Trans fatty acids (infra-red analysis)	32.8
Linoleic acid	.0
Linolenic acid	.0
Oleic acid (by diff.)	46.3

weekly. The results are shown in Table II.

Results. It is apparent from a consideration of the weight increments of the rats at various time intervals during the assay period, that there is no interference with growth as a result of the administration of 250 mg or 500 mg of a hydrogenated triolein to the animals in the various groups. In fact, with animals receiving no other fat in the diet, supplementation with this hydrogenated triolein product results in a slight gain in weight over the 8-week period (Groups II and III, Table II) as compared with the animals continued on the fat-free ration (Group I). The gains in weight of the groups in which both linoleate and hydrogenated triolein are fed to the animals are remarkably similar; at the lower level of linoleate at the end of the 8-week assay period the weight increment was 65.6 g when the linoleate was fed alone, and 63.6 g and 63.8 g when either 250 mg or 500 mg of hydrogenated triolein were administered concomitantly (Groups IV, V, VI). The weight gain at the higher level of linoleate was 73.4 g, and 70.8 g and 81.1 g when the 2 levels of hydrogenated triolein were given concomitantly (Groups VII, VIII, IX). These studies demonstrate that the trans isomers of oleic acid in the hydrogenated triolein have no antimetabolite activity for the essential fatty acids either in the presence or absence of linoleic acid.

When animals are placed on either fat-free diets alone or on fat-free diets containing additives which are known to deplete more rapidly the essential fatty acid stores of an animal, it is conceivable that the further addition of any substance which does not possess essential fatty acid activity, but which might

TABLE II. Effect of Various Supplements over an 8 Week Period on the Growth of Male Rats Previously Depleted of Essential Fatty Acids.

Group	Supplement*		No. of animals	Avg wt at start (g)	Wt increment (g) after		
	T	L			3 wk	6 wk	8 wk
I			15	232	- 15.2	- 7.5	- .3
II	250		11	238	- 5.4	+ 8.9	+ 14.8
III	500		10	237	- 11.3	- 5.9	+ 2.0
IV		20	11	242	+ 16.6	+ 42.1	+ 65.6
V	250	20	11	235	+ 19.4	+ 45.4	+ 63.6
VI	500	20	13	250	+ 19.9	+ 46.8	+ 63.8
VII		50	9	249	+ 22.6	+ 51.1	+ 73.4
VIII	250	50	8	238	+ 26.4	+ 49.6	+ 70.8
IX	500	50	9	232	+ 26.9	+ 60.4	+ 81.1

* T = Hydrogenated triolein; L = Methyl linoleate.

ordinarily be used efficiently as a nutrient, might lead to an accentuation of the essential fatty acid deficiency symptoms. In fact, addition of certain growth factors might induce a more complete deficiency of essential fatty acids due to production of certain stresses. Experiments performed in our laboratories have shown that animals deficient in essential fatty acid accumulate cholesterol esters and total lipid in the liver leading to a typical "fatty" liver condition(12). The subsequent administration of the methyl ester of essential fatty acid, linoleic, alleviates this condition within 4 weeks. Supplementation with methyl oleate for the same length of time, however, not only does not reduce the cholesterol levels in the liver but tends to accentuate the accumulation of both the cholesterol esters and the total lipid in this organ.§ In view of the fact that oleic acid does not possess essential fatty acid activity, this result is not surprising. To label as antimetabolites or as antagonists substances which do not alleviate the deficiency symptoms in an animal caused by a lack of essential fatty acids is certainly to be avoided. It must be demonstrated that these substances increase the requirement for essential fatty acids or in the absence of the essential fatty acids aggravate the deficiency syndrome. The trans isomers, the oleic acid and the stearic acid in the hydrogenated triolein are not anti-metabolites for the essential fatty acids according to these criteria.

Summary. Rats which were depleted of essential fatty acids after 16 weeks on a fat-free diet were maintained for 8 weeks

thereafter on either a continued fat-free diet, fat-free diets containing 2 levels of hydrogenated triolein (containing 33% of trans isomers and no essential fatty acids), fat-free diets containing 2 levels of methyl linoleate, or fat-free diets supplemented with both linoleate and the hydrogenated triolein at the 2 levels. The animals receiving the linoleate supplement alone or the linoleate plus the hydrogenated triolein showed equivalent weight gains over an 8-week period. In the absence of the linoleate there was no aggravation of the deficiency syndrome. No observable antimetabolic activity of the hydrogenated triolein was evident.

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Effect of Antimetabolites on Growth of *Endamoeba histolytica*. III. 5,6-dimethylbenzimidazole and Related Compounds.* (23275)

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Hendlin and Soars(1) have studied effects of 5,6-dimethylbenzimidazole and related compounds on the growth of *Lactobacillus lactis* Dorner. They reported that 5,6-dimethylbenzimidazole, 1,2-dimethyl-4,5-diaminobenzene, and several substituted benzimidazoles inhibited vit. B₁₂-requiring microorganisms. The toxicity of the diamine was competitively antagonized by B₁₂. However, such was not the case with the 5,6-dimethylbenzimidazole and its analogues. Oginsky and Smith(2) showed that B₁₂ stimulates rate of acetate oxidation by resting suspensions of *Escherichia coli* (strains which require this vitamin), whereas no stimulation occurred with benzimidazole, 2,5-dimethylbenzimidazole, and 5,6-dimethylbenzimidazole. On the other hand, Woolley(3,4) recognized 1,2 dimethyl-4,5-diaminobenzene as a probable precursor of B₁₂ so an antimetabolite (1,2-dichloro-4,5-diaminobenzene) of this compound was synthesized. Dichloro-diaminobenzene was toxic to microorganisms which did not exhibit a nutritional need for B₁₂. This inhibition by the antimetabolite was competitively reversed by the dimethyl-diaminobenzene. Although a slight stimulatory activity on *Endamoeba histolytica* was observed using crude preparation of B₁₂(5), additional studies with pure crystalline B₁₂ have suggested that this vitamin is not a specific requirement for the amebas; in fact, evidence has accumulated that *E. histolytica* is capable of synthesizing B₁₂.

The present studies were conducted in order to determine the effect of 5,6-dimethylbenzimidazole and related compounds on *E. histolytica* so that we may learn something about vit. B₁₂ synthesis in the amebas.

Materials and methods. The NRS strain of *E. histolytica* was used. Stock cultures of this strain growing with a mixed bacterial flora were maintained on a modified Ringer-egg-serum medium. However, the effects of the compounds were studied in ameba cultures containing no viable bacteria. Detailed technics employed have been described(6,7,8).

The following compounds were studied: 5,6-dimethylbenzimidazole, 1,2-dimethyl-4,5-diaminobenzene (obtained from Dr. David Hendlin, Research Laboratories, Merck and Co., Rahway, N.J.), 2,5-dimethylbenzimidazole, 4,5-diphenyl-2-imidazolone, 5-methylbenzimidazolone (obtained from Dr. Gustav J. Martin, Research Laboratories, National Drug Co., Philadelphia, Penna.), 1,2-dichloro-4-nitro-5-hydroxybenzene, 1,2-dichloro-5-sulfanilamido-5-aminobenzene, and 1,2-dibromo-4,5-diaminobenzene (obtained from Dr. D. W. Woolley, Rockefeller Institute for Medical Research, New York City). These compounds were tested in concentrations ranging from 0.001 mg/ml to 1 mg/ml. In cases where the compounds inhibited *E. histolytica*, reversal studies were performed with crystalline vit. B₁₂ thymidine (Nutritional Biochemicals Corp., Cleveland, O.), and folic acid (from Dr. J. M. Rueggesser, Lederle Laboratories Division, Am. Cyanamid Co., Pearl River, N.Y.)

Results. The activities of the compounds tested are listed in Table I. Only 1,2-dimethyl-4,5-diaminobenzene was without inhibitory activity against the amebas; instead this compound acted as a growth stimulant to the amebas. The inhibitory activities of 5,6-dimethylbenzimidazole, 2,5-dimethylbenzimidazole, 1,2-dichloro-4-sulfanilamido-5-aminobenzene, and 1,2-dibromo-4,5-diaminobenzene were reversed by B₁₂ (0.001 mg/ml) and 1,2-dimethyl-4,5-diaminobenzene (0.1 mg/ml). Folic acid (0.1 mg/ml) and thymidine (0.1

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TABLE I. Effect of 5,6-Dimethylbenzimidazole and Related Compounds on *In Vitro* Growth of *Endamoeba histolytica* under Bacteria-Free Conditions.

Compounds assayed	Stimulatory to amebas	Amebocidal activity	Minimum conc. amebocidal, mg/ml	Compounds able to reverse inhibition	Compounds unable to reverse inhibition
5,6-dimethylbenzimidazole		+	.1	DMDAB (partial), B ₁₂ (partial)	Folic acid, thymidine
1,2-dimethyl-4,5-diaminobenzene (DMDAB)	+				
2,5-dimethylbenzimidazole		+	.1	<i>Idem</i>	<i>Idem</i>
4,5-diphenyl-2-imidazolone		+	1	Folic acid (partial), thymidine (partial)	DMDAB, B ₁₂
5-methylbenzimidazolone		+	1		Folic acid, thymidine, DMDAB, B ₁₂
1,2-dichloro-4-nitro-5-hydroxybenzene		+	1		<i>Idem</i>
1,2-dichloro-4-sulfanilamido-5-aminobenzene		+	.5	DMDAB (complete), B ₁₂ (complete)	Folic acid, thymidine
1,2-dibromo-4,5-diaminobenzene		+	.5	<i>Idem</i>	<i>Idem</i>

mg/ml) were ineffective in reversing this inhibition. Reversal of 5-methylbenzimidazolone and 1,2-dichloro-4-nitro-5-hydroxybenzene was not possible when folic acid, thymidine, B₁₂, or dimethyldiamine were added. Interestingly, the inhibitory activity of 4,5-diphenyl-2-imidazolone was reversed with folic acid and thymidine but not by B₁₂ or the dimethyldiamine.

Discussion. It appears from these data that 1,2-dimethyl-4,5-diaminobenzene is a precursor of vit. B₁₂ in *E. histolytica*. The amebas, apparently, are able to convert this intermediate to B₁₂, yet are unable to accomplish the total synthesis of this dimethyldiaminobenzene since this substance added to bacteria-free amebas produced a great increase in amebic growth and multiplication. 5,6-dimethylbenzimidazole is thought by some workers to be a precursor of B₁₂ especially in view of the fact that it has been identified as an acid degradation product of B₁₂(9). On the other hand, Emerson *et al.*(10) found this compound to be without B₁₂ activity for *L. lactis* Dorner. Furthermore, Oginsky and Smith(2) reported that 5,6-dimethylbenzimidazole inhibited the ability of B₁₂ to stimulate the rate of oxidation of acetate in *E. coli*. This compound also inhibited *L. lactis*(1); the dimethyldiamine and its ribityl analogue inhibited *Euglena gracilis*(11). Although the

mechanism of this inhibition is not clearly understood at this time, Hendlin(12) suggests that the inhibitory effect of 5,6-dimethylbenzimidazole might result from its interference with adsorption or absorption of the vitamin.

The fact that the inhibition is reversed by B₁₂ and also by the dimethyldiamine indicates that this compound, the dimethylbenzimidazole, prevents the utilization of B₁₂ in the amebas or prevents the conversion of B₁₂ precursors into the vitamin.

The inhibition observed by 2,5-dimethylbenzimidazole, 1,2-dichloro-4-sulfanilamido-5-aminobenzene and 1,2-dibromo-4,5-diaminobenzene may well have been due to the blockade of conversion of the dimethyldiamine to B₁₂ since reversal was possible by supplying the precursor or the vitamin. Other workers have also reported on the inhibitory activity of 2,5-dimethylbenzimidazole(10). These substituted benzene compounds are possibly antimetabolites of the dimethyldiamine as postulated by Woolley(3,4,13).

Since reversal of inhibition was not possible with folic acid and thymidine, it may be surmised that, in *E. histolytica* at least, B₁₂ does not function in thymidine synthesis. The logic here is that if a vitamin acts in a cell at some stage in the synthesis of essential metabolites, then it is to be expected that addition

to the growth medium of the preformed metabolite would abolish the need for the vitamin.

The mode of action of 4,5-diphenyl-2-imidazolone is not understood; the data suggest that the activity may have been due primarily to interference of folic acid metabolism (or some function of folic acid) or in the utilization of thymidine. Neither B₁₂ nor the dimethyldiamine were able to reverse the toxicity due to this compound.

Why the inhibition due to 5-methylbenzimidazolone and 1,2-dichloro-4-nitro-5-hydroxybenzene did not fit the pattern of the dimethylbenzimidazole is not clear. These compounds were toxic to amebas at higher concentrations and large quantities of B₁₂ or dimethyldiamine did not reverse the inhibition. Perhaps the toxicity in these cases was due to other than antimetabolite effects.

The importance of vit. B₁₂ in *E. gracilis* has been thoroughly reviewed(14,15). *Tetrahymena*, on the other hand, is able to synthesize adequate amounts of vit. B₁₂(16).

We are aware of the fact that there has been reported a substance in egg yolk which is said to bind B₁₂(17), however, in view of the fact that B₁₂ added to egg slant media used in these experiments released the inhibition of amebas, it is probably not interfering with our assay method.

Summary. 1,2-dimethyl-4,5-diaminobenzene was highly stimulatory to *Endamoeba histolytica* under bacteria-free conditions. 5,6-dimethylbenzimidazole, 2,5-dimethylbenzimidazole, 1,2-dichloro-4-sulfanilamido-5-aminobenzene and 1,2-dibromo-4,5-diaminobenzene were inhibitory to the amebas; the inhibition was overcome by either vit. B₁₂ or the dimethyldiamine. 5-methylbenzimidazolone and 1,2-dichloro-4-nitro-5-hydroxyben-

zene were also inhibitory to the amebas, but reversal was not possible with B₁₂, dimethyldiamine, folic acid, or thymidine. It is suggested that the dimethyldiaminobenzene is a precursor of vit. B₁₂ in *E. histolytica* and that the dimethylbenzimidazole and chloro- and bromo-substituted benzenes are antimetabolites of this precursor.

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Distribution and Excretion of Hexafluorophosphate. (23276)

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(Introduced by Olaf M'c'ensen)

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In a recent study of the metabolism of various complex fluorides(1), it was shown that administration of potassium hexafluorophosphate to rats resulted in no increase in fluoride content of the skeletal and dental tissues, and had no effect on either incidence or severity of dental caries. These findings, together with evidence attesting to the low toxicity of hexafluorophosphate in both the frog and rabbit(2), indicate that this anion is relatively, if not completely, inert. While at least one other metabolically inactive complex inorganic fluoride (KBF_4) has been reported(3), the physiological properties of PF_6^- are of some theoretical interest in that it represents, to our knowledge, the only phosphorus-containing anion* which is not readily metabolized. It was decided, therefore, to extend information relative to its metabolism by investigating the occurrence of PF_6^- in tissues other than those of bone and teeth, and to determine the mode of its excretion.

Experimental. Preparation of labelled hexafluorophosphate†. KP^{32}F_6 was synthesized according to Woyski(5) using PCl_5 prepared from radioactive phosphorus(6,7):

$\text{KCl} + \text{P}^{32}\text{Cl}_3 + 6\text{HF} \rightarrow \text{KP}^{32}\text{F}_6 + 6\text{HCl}$
(in liquid HF). After recrystallization from hot water, the percentage composition of the product was: P, 16.75; F, 61.57; free F ion, 0.18. Theoretical for KPF_6 : P, 16.83; F, 61.93. Radioactivity was determined by conventional procedures using an end-window counter, and the values obtained were corrected for self absorption. Fluoride was determined according to Willard and Winter(8) as modified by McClure(9). *Collection*

* PF_6^- is also unique in that it is the only compound of pentavalent phosphorus known to exist as the free anion(4).

† Neutron irradiation of the commercially available ammonium salt (Ozark-Mahoning Co., Tulsa, Okla.) may offer a more feasible method of preparing radiophosphorus-tagged PF_6^- .

of excreta. Metabolism cages were constructed to permit separate collection of urine and feces. A series of holes $5\frac{1}{2}$ " in diameter were cut in a wood board, and upholsterers' tacks inserted on $\frac{1}{2}$ " centers around the perimeter of each. Using the tacks as a frame, nylon cord was strung to form a screen floor beneath each opening.‡ An inverted 2 liter beaker provided with a $3/8$ " hole to admit a drinking tube served as a cage. For stability, scotch tape was placed across the top of the water bottle and fastened to the board on either side. A strip of filter paper was attached to the back of the board, angulated approximately 45° to horizontal, and fastened to the edge of the work bench. The free edge was folded back to form a collection trough for the feces. Urine was trapped by absorption on the paper. Female Sprague-Dawley rats weighing 100-120 g were employed throughout. Twenty-seven rats were injected intraperitoneally with 1 mg of KP^{32}F_6 in 1 ml of distilled water, and placed in separate metabolism cages for 1, 3, 6, 12, or 24 hours. At the end of the collection periods each rat was induced to empty its bladder by digital pressure applied at the base of the tail. The filter paper was placed under ultraviolet light and the strongly fluorescing urine spots cut out. These were combined with the nylon cord and both extracted with hot distilled water. Recovery of labelled phosphorus added as PF_6^- to 3 control urine papers was $97.0 \pm 1.3\%$ (mean \pm S.E.). An additional 42 rats were injected with 1 mg of KP^{32}F_6 as before and sacrificed by decapitation at the end of 5, 10, 15, and 30 minutes, and 1, 2, 6, and 18 hours. The animals were bled individually into oxalated beakers and the whole blood and plasma analyzed for

‡ Rats have been retained up to one week in this type of cage. For retention over longer periods of time, stainless-steel wire may be substituted for the nylon cord.

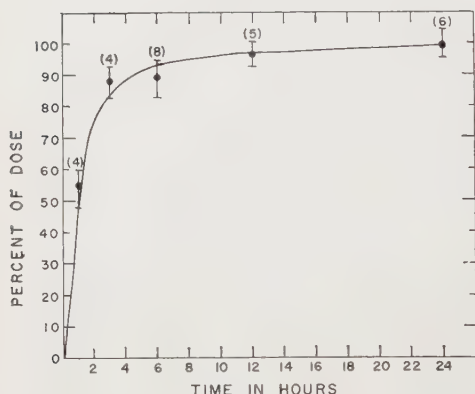
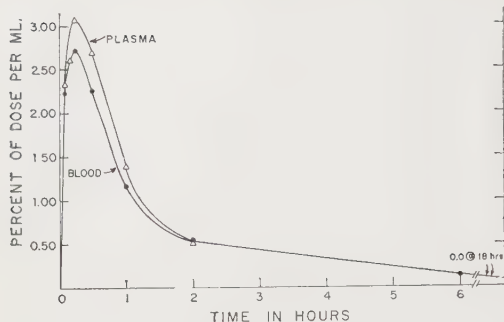


FIG. 1 (top). Conc. of radiophosphorus in blood and plasma following intraper. inj. of KP^{32}F_6 .

FIG. 2 (bottom). Excretion of radiophosphorus in urine following intraper. inj. of KP^{32}F_6 .

radiophosphorus. Two rats were injected intravenously with 1 mg of KP^{32}F_6 dissolved in 0.2 ml of physiological saline, and sacrificed by decapitation at the end of 10 minutes. The heart, liver, lungs, kidneys, spleen, and a portion of thigh muscle were excised, blotted, and weighed. The samples were ignited for 3 hours at 550°C , dissolved in 1N HCl, and analyzed for radiophosphorus. Recovery of labelled phosphorus added as PF_6^- to 6 control tissue samples was $95.9 \pm 1.6\%$ (mean \pm S.E.).

Results. Concentration of radioactivity in whole blood and plasma reached a maximum at 15 minutes, then fell precipitously (Fig. 1). No detectable activity was found in the blood 18 hours after injection. Concentration values were consistently higher in plasma than in blood, and may reflect differences in water content. The findings are consistent with the belief that blood cells are freely permeable to the PF_6^- ion.

Ten minutes after intravenous injection the mean concentration of radiophosphorus in the tissues, expressed as percent of dose per gram of fresh weight, was: liver, 0.57; spleen, 0.55; heart, 0.51; lungs, 0.97; kidney, 1.67; and muscle, 0.29.

The high radiophosphorus content of kidney seems in accord with the role of this organ in the excretion of the label. Thus 55% of the dose had been cleared at the end of one hour, and 88% by the end of 3 hours (Fig. 2). The 24-hour urine contained all of the injected radiophosphorus. The rapidity of clearance suggests that tubular reabsorption was minimal, if not absent.

In 8 urine samples selected at random, the ratio of calculated-to-found fluoride was 0.98 ± 0.03 (mean \pm S.E.). This finding substantiates the physiological stability of PF_6^- . Similarly, the quantitative recovery of injected radiophosphorus in urine can only be explained by the inability of the rat to hydrolyze PF_6^- to PO_4^{3-} and F^- .

The physiological stability of this ion, coupled with its quantitative excretion in the urine, suggests consideration of radiophosphorus-tagged hexafluorophosphate as a possible tool in renal function studies.

Summary. 1. Distribution and excretion of radiophosphorus labelled hexafluorophosphate ion has been investigated in the rat. 2. The concentration of PF_6^- in whole blood and plasma reached a maximum at 15 minutes, fell precipitously, and none was found 18 hours after its injection. 3. Evidence is presented that PF_6^- is not hydrolyzed by the rat. 4. Injected PF_6^- is rapidly and quantitatively excreted in the urine.

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Pharmacodynamics of Chlorothiazide (Diuril), An Orally Effective Non-Mercurial Diuretic Agent.* (23277)

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The following report is concerned with chlorothiazide (Diuril)[†] a diuretic agent of low toxicity, which is well absorbed by the oral route(1) and is equally as potent as meralluride (given parenterally).

Methods and materials. The acute renal hemodynamic responses to doses up to 10 mg/kg of chlorothiazide (Diuril) given intravenously were observed in anesthetized dogs employing methods and technics described previously(2). The acute effect on water and electrolyte excretion was also observed in unhydrated dogs(3).

The clinical studies were done on 10 male patients who had been in mild heart failure but who at the time of the study were free of edema. The technic for evaluation of diuretic agents has been described previously(4,5). The subjects of the study were ambulatory, hospitalized patients maintained on a metabolic ward, eating a diet containing 50 mEq of sodium per day, and drinking 3,000 ml of distilled water per day. After a suitable period of adjustment to the diet the patient's urinary sodium was approximately 90% of the dietary intake, *i.e.*, about 45 milliequivalents per 24 hours. When this point was reached, the drug was given as a single dose at 6 a. m. and observations were made of subjective and objective signs of toxicity as well as of the urinary sodium excretion, analyzed

with the use of a Beckman flame photometer. After another suitable interval (5 to 7 days) for equilibration, the next dose was given and so forth. The drug was administered 10 times at each dose and the results subjected to appropriate statistical analysis as justified by previous studies(4).

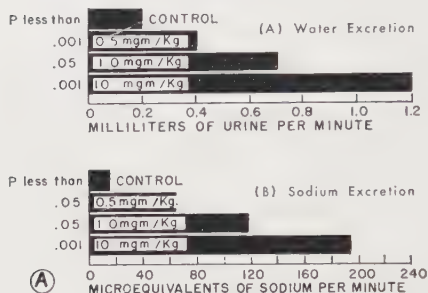
Results. Following intravenous administration of chlorothiazide (Diuril) to dogs there was a significant increase in water excretion associated with an increase in sodium, potassium, and chloride excretion which was generally proportional to the dose (Fig. 1A and 1B). The diuretic threshold dose was less than 0.5 mg/kg body weight. The diuretic effect was not due to an increase in glomerular filtration rate since renal blood flow and glomerular filtration rate were not increased (Fig. 2). This leads to the conclusion that the increase in water and electrolyte excretion is due to a direct tubular effect of the chlorothiazide which blocks the reabsorption of these substances. Mean blood pressure was not altered.

The particular electrolyte excretion pattern associated with administration of a single 2000 mg oral dose of chlorothiazide to a typical patient is presented in Fig. 3. The greatest increase in cations was in the excretion of sodium but potassium excretion was also increased temporarily. There was a moderate decrease in ammonia excretion following administration of the drug. Among the anions observed, the greatest increase in excretion rate was in chloride with a significant but temporary increase in bicarbonate. Total in-

* Supported in part by Houston Heart Assn.

[†]6-chloro-7-sulfamyl-1,2,4 - benzothiadiazine-1, 1-dioxide. Supplied as Diuril by Merck, Sharp & Dohme, West Point, Pa.

RESPONSE IN WATER AND SODIUM EXCRETION
FOLLOWING CHLOROTHIAZIDE ADMINISTRATION IN DOGS
(Mean Values)



RESPONSE IN POTASSIUM AND CHLORIDE EXCRETION
FOLLOWING CHLOROTHIAZIDE ADMINISTRATION IN DOGS
(Mean Values)

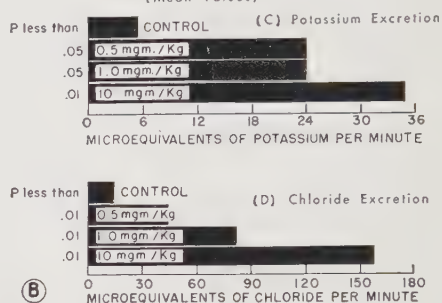


FIG. 1 (A & B). Responses in excretion of water, sodium, potassium, and chloride are proportional to dosage of the drug admin. to dogs intrav.

crease in excretion of bicarbonate per 24 hours was of a small order (Fig. 3).

The response to oral administration as compared to the same dose administered intravenously was observed in several patients. The effect on sodium excretion in one such study is presented in Fig. 4. There was a greater initial response following intravenous administration but following oral administration the response persisted for a longer period of time than it did after the intravenous route. Consequently, total increase in excretion of sodium over a 24-hour period was greater after the oral route of drug administration.

A summary of the natriuretic response to 2000 mg of chlorothiazide given to 10 patients is presented in Table I. Doses in excess of this amount of drug produced very

little additional diuresis or natriuresis.

There was no significant toxicity noted in the patients who received doses up to 2000 mg per day. Nausea and vomiting were not observed. Two patients complained of mild weakness and lethargy.

Discussion. Chlorothiazide is an effective orally administered diuretic agent which is a non-mercurial and apparently is not toxic. It is a potent inhibitor of the renal tubular reabsorption of sodium. It causes a minimal increase in excretion of bicarbonate (Fig. 3) and a considerable increase in excretion of chloride. An oral dose has a rapid onset of action (2 hours) and a short duration (12 hours) of effect. In current trials, it maintains its effectiveness when administered daily (in contrast to carbonic anhydrase inhibitors). It is slightly more effective after oral than after intravenous administration (Fig. 4) indicating complete or at least adequate absorption by the oral route. A 2 dose daily schedule is optimal. The maximum effect appears to be achieved at a dose of 2000 mg daily (1000 mg at breakfast and supper). No toxicity was observed in 20 patients who received the drug daily for 3 months. Calculation of "potency" (4,5) reveals that it is equivalent to 1 cc of meralluride (Mercurhydrin) given intramuscularly (Fig. 5) at a dose of slightly more than 1000 mg administered orally and at a dose of 2000 mg given orally in 2 divided doses, it is nearly as potent as 2 cc of meralluride (Fig. 5).

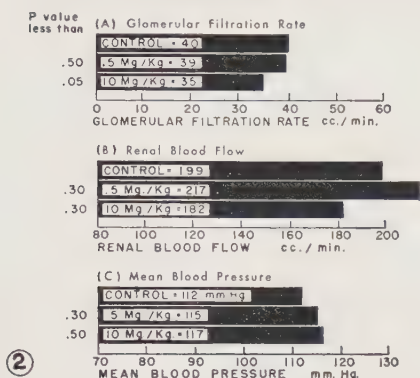
In comparing chlorothiazide to other oral diuretic agents (Fig. 5) at the maximum clinical

TABLE I. Response of 10 Patients in Sodium Excretion following Oral Administration of 2000 mg of Chlorothiazide (mEq/24 Hr).

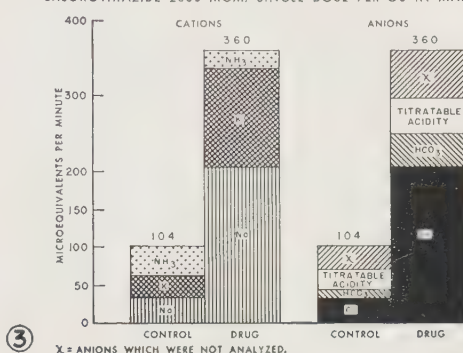
	Control	Drug	Increase
	43	167	124
	41	138	97
	45	162	117
	48	145	97
	42	174	132
	45	130	85
	47	147	100
	46	142	96
	41	143	102
	47	137	90
Avg	45	149	104
P value*			<.001

$$* t = \frac{\bar{x} - \mu}{\sqrt{\frac{Sx^2}{n(n-1)}}}$$

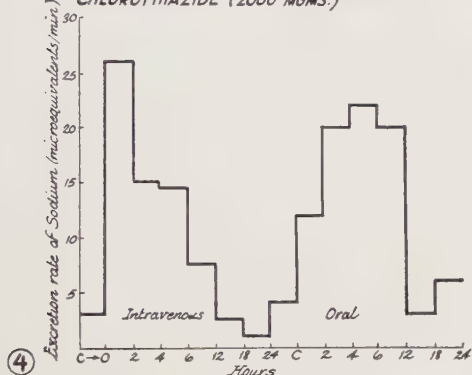
RENAL HEMODYNAMIC EFFECTS OF CHLOROTHIAZIDE



RESPONSE IN ELECTROLYTE EXCRETION FOLLOWING CHLOROTHIAZIDE 2000 MG/M. SINGLE DOSE PER OS IN MAN



COMPARISON OF ORAL AND INTRAVENOUS CHLOROTHIAZIDE (2000 MG/M.)



COMPARATIVE POTENCY OF ORAL AND PARENTERAL DIURETIC AGENTS

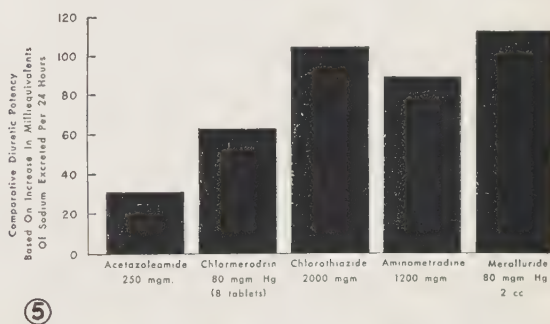


FIG. 2. When chlorothiazide is admin. to dogs intrav., there is no significant effect on renal hemodynamics.

FIG. 3. Electrolyte excretion pattern following oral admin. of chlorothiazide to man is characterized mainly by increase in excretion of sodium and chloride with lesser effects on potassium, bicarbonate and titratable acidity.

FIG. 4. Comparison of response to chlorothiazide given orally and intrav. Immediate response in sodium excretion after intrav. admin. was greater but total 24 hr increase in sodium was greater after oral admin.

FIG. 5. In producing an increase in excretion of sodium (as a measure of potency) chlorothiazide appears to be more powerful than chlormerodrin (Neohydrin) and acetazolesamide (Diamox). Doses larger than 250 mg of Diamox produce no greater effect. Doses in excess of 80 mg (8 tablets) of Neohydrin produce diarrhea or nausea in over 25% of the patients. A dose of 2000 mg of chlorothiazide is nearly as potent as 2 cc (equivalent to 80 mg Hg) of meralluride (Merehydrin) given intrav.

cally tolerated doses and employing the technique of potency estimation as previously described(4,5), it appears to be more potent in causing an increase in sodium excretion than chlormerodrin (Neohydrin) and acetazolesamide (Diamox).

Conclusions. Chlorothiazide at a daily dose of 2000 mg (500-2000 mg range) is effective as an oral diuretic agent with no demonstrable toxicity.

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Support of Water Absorption by Rat Jejunum *in vitro* by Glucose in Serosal Fluid. (23278)

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The relationship between glucose and absorption of water by the rat small intestine *in vitro* exhibits some interesting and puzzling features. Water transport is negligible unless glucose is added to the bathing fluid, and no other substance has thus far been found which can adequately substitute for glucose in this respect(1,2,3). Possibly in the absence of added glucose, the mucosa has no substrate to provide the energy to sustain water transport; yet rat small intestinal mucosa has been reported to have a high endogenous respiration(4) and can transport at least methionine against a concentration gradient without added glucose(5). It has also been reported that to support water absorption in surviving rat intestine, the glucose must be supplied from the mucosal fluid; glucose added to the serosal medium (up to 2% in concentration) did not appreciably augment the water absorption occurring with glucose in the lumen or promote water absorption when mucosal medium is glucose-free(2). Yet *in vivo*, water absorption proceeds from glucose-free solutions placed in the intestine, and the normal route of entry of blood glucose into mucosal cells is presumably through their serosal poles. Failure of glucose in the serosal fluid to stimulate water-absorption in the surviving intestine has been taken to indicate that the non-mucosal tissues present a barrier to diffusion of glucose which cannot be demonstrably overcome even when the glucose concentration is more than 20 times that normally present in the body fluids. On the other hand, since glucose is absorbed by the surviving intestine from the mucosal to the serosal fluid, the diffusion barrier is certainly not absolute. Hence one would expect that by increasing sufficiently the concentration in the fluid bathing the serosa, the mucosa could be supplied with enough glucose to bring about water absorption. Otherwise, leaving secondary effects aside, one would be forced

to consider some rather implausible explanations for the observations. It therefore appeared important to examine the relationship between water absorption and glucose concentration in the fluid bathing the serosa of the rat surviving intestine.

Methods. The procedure of Fisher & Parsons(6) was followed with minor modifications. Unfasted male albino rats 200-400 g were employed, and ether was the anesthetic. Jejunal loops ranging in length from 30-55 cm were used. Krebs bicarbonate-Ringer fluid was made up according to Umbreit *et al.* (7). The rinsing fluid was 155 mM NaCl. The initial volume of the mucosal fluid was 25 ml; that of the serosal fluid, 40 ml. Whenever the composition of the serosal medium was altered, an isosmotic solution of the new solute was substituted for an equivalent volume of isotonic NaCl, so that initially the fluids bathing both sides of the intestine were always isosmotic (approximately 300 mM per K of water). All fluids were gassed with 5% CO₂ in O₂ at 38° before use. The experiments were carried out at 38°C; and 5% CO₂ in O₂, saturated with water vapor at the same temperature, was employed for aerating and mixing the fluids. The absorption time was 1 hour and the distension pressure was about 20 cm of water. Water absorption was calculated from the sum of the changes in weight in the serosal fluid and the intestinal wall. Based on control experiments, a small correction was made for the combined effects of evaporation or condensation, incomplete drainage of the chamber, and excess fluid adhering to the loop. The final water content of the tissue was estimated by slitting the loop open longitudinally, thoroughly blotting it on filter paper and drying it to constant weight at 100°C. Initial water content was measured on a series of loops taken down at what would otherwise have been the beginning of the absorption period.

TABLE I. Effect of Increasing Glucose Concentration in Isosmotic Serosal Fluids on Water Absorption when Mucosal Fluid Contains Approximately 1/10 Isosmotic Glucose.

Glucose in serosal fluid, mg %	Approx. fraction of osmotic pressure contributed by glucose	No. of exp.	Water absorption, $\mu\text{l}/\text{cm}/\text{hr}$
0	0	4	122 ± 13
530	1/10	11	124 ± 4
2660	1/2	8	124 ± 10
4100	4/5	4	92 ± 2

Results. The data in Table I confirm the observations of Fisher(2) that provided the mucosal fluid contains adequate amounts of glucose (customarily an initial concentration of some 500-600 mg%), additional glucose up to 1/2 isosmotic concentration in the outer fluid does not appreciably affect water absorption. When the serosal fluid contained 4/5 isosmotic glucose, the water absorption was reduced from $124 \pm 10 \mu\text{l}/\text{cm}/\text{hr}$ (standard error of the mean) to $92 \pm 2 \mu\text{l}/\text{cm}/\text{hr}$.

The effects of adding glucose to the serosal fluid when the mucosal fluid was glucose-free are shown in Fig. 1. With increasing glucose in the serosal fluid, the rate of water absorption increased approximately linearly from a slightly negative value (which is probably real and possibly due to secretion)* until the outer fluid contained 1/2 isosmotic glucose. At this point however, the average rate ($81 \mu\text{l}/\text{cm}/\text{hr}$) was still only 65% of that measured when glucose was present only in the mucosal fluid ($124 \mu\text{l}/\text{cm}/\text{hr}$), and no further augmentation in absorption occurred when the serosal glucose concentration was raised until all the NaCl in the Ringer's was replaced by glucose.

Water absorption did not occur when galactose, xylose, or mannitol was substituted for glucose in the serosal fluid (Table II). To this extent, the stimulation of water absorption was specific for glucose. Galactose and xylose can penetrate the mucosal cells, at least from the luminal pole, since they can be absorbed(8,9), and galactose can prob-

ably be utilized to some extent(8). Mannitol is not appreciably absorbed from the rat intestine *in vivo*(10), and presumably would be confined to the extracellular spaces.

Additional observations. With 5% CO₂ in N₂ instead of 5% CO₂ in O₂ used as the aerating gas, and with 1/2 isosmotic glucose in the serosal fluid, water absorption was reduced by 80% (2 experiments). When bicarbonate was omitted from the mucosal fluid, significant water absorption did not occur (5 experiments; pH in lumen 4.85). Reducing the Ca in the Ringer's fluid by one-half (*i.e.* to 1.25 mM) had no effect on water absorption (3 experiments). Substitution of acetate or glycerol for glucose in both fluids did not stimulate water absorption (2 experiments each).

Discussion. Glucose supplied from the serosal fluid can apparently support water absorption from the surviving rat small intestine. The large concentration of glucose in the serosal bathing medium which is required to yield maximum water absorption is no doubt indicative of a high barrier either to diffusion of glucose from the serosa to the mucosal cells or to permeation into them, or both. To account for the finding that this maximum rate of water absorption is only about 65% of that observed when glucose is added to mucosal fluid, at least two explanations suggest themselves. The first is that, due to the diffusion barrier, it takes time for the establishment of an adequate glucose supply to the mucosa from the serosal fluid, so that even with large serosal fluid concentrations, the mucosa is operating with optimal glucose over only a fraction of the experimental period. Moreover, as soon as water absorption is initiated "solvent drag" would tend to oppose the glucose diffusion stream. On the other hand, with glucose in the lumen,

TABLE II. Effect of Isosmotic Outer Fluids Containing 1/2 Isosmotic Non-Electrolyte. Mucosal fluid glucose-free Krebs bicarbonate-Ringer.

Non-electrolyte in serosal fluid	No. of exp.	Water absorption, $\mu\text{l}/\text{cm}/\text{hr}$
Glucose	11	81 ± 6
Galactose	4	-1 ± 2
Xylose	4	-8 ± 1
Mannitol	4	-2 ± 2

* It is interesting in this connection that Wilson has recently reported water absorption by sacs of everted hamster jejunum incubated in sugar-free solutions(11).

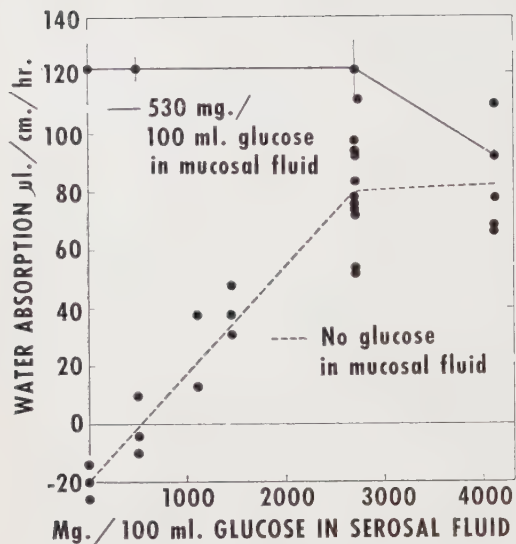


FIG. 1. Effect of increasing glucose concn. in serosal fluid on water absorption.

the substrate becomes immediately available to the mucosal cells.[†] Although increasing the serosal glucose level from $\frac{1}{2}$ isosmotic to nearly $\frac{4}{5}$ isosmotic fails to increase water transport further, this failure may be due to secondary deleterious effects associated with the extreme change in the composition of Ringer medium. Thus, with $\frac{4}{5}$ isosmotic glucose in the serosal fluid, the rate of water absorption from glucose containing mucosal fluid is decreased, and there is no significant difference in water transport whether or not glucose is present in the mucosal fluid (Fig. 1).

While diffusion time must play a role, other factors might also be of importance. A second possible explanation for the observation under discussion is that absorption of glucose in some way carries water with it. It may be, in the limiting case, that the rate of isosmotic absorption of water and solutes other than glucose is the same whether glucose is provided from the mucosal or serosal fluids, but that with glucose present in the lumen, the absorbed glucose takes with it ad-

ditional water to maintain osmotic equilibrium. An attempt was made to obtain evidence on whether such a factor was operating. With $\frac{1}{2}$ isosmotic glucose in the serosal fluid 2 other absorbable sugars, galactose or 3-methyl glucose, were used in the mucosal fluid instead of glucose. With galactose the observed rate of H_2O absorption was $94 \pm 8 \mu\text{l}/\text{cm}/\text{hr}$, which is $13 \mu\text{l}/\text{cm}/\text{hr}$ greater than the value of $81 \pm 6 \mu\text{l}/\text{cm}/\text{hr}$ found when the mucosal fluid was Ringer's; but the difference is not statistically significant. An average of 1.6 mg of galactose/cm/hr was absorbed which could have accounted for $\frac{1.6}{54} \times 1000 = 30 \mu\text{l}$ of isosmotic H_2O /cm/hr.

Essentially similar findings were obtained with 3-methyl glucose. The results were thus no more than suggestive.

Summary. Glucose in the Ringer's fluid bathing the serosal surface supports water absorption by isolated jejunal loops of rat intestine. The rate of water absorption increased with increasing glucose concentration up to $\frac{1}{2}$ isosmotic. This maximum rate was about 65% of that found when $\frac{1}{10}$ isosmotic glucose was present in the Ringer's fluid bathing the mucosa.

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[†] The endogenous carbohydrate stores of mucosal cells appear to be very small. Total glycogen content of 4 typical jejunal loops excised and taken immediately for analysis(12) ranged from 0.6 to 2.2 mg.

Rate of Collagen Formation in Biopsy-Connective Tissue of the Rat. (23279)

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The rate of collagen formation has been difficult to determine in man and animal. Recently, collagen formation has been studied in granulomatous lesions induced by the injection of an irritant, such as carrageenin(1). When the sponge implantation technic(2) is used for isolation of connective tissue, the age of the newly developed connective tissue is known and rate of collagen formation may be quantitated. Tissue response to the plastic sponge implant has been followed in the albino rat histologically and biochemically, and the accumulated data indicate a predictable and reproducible reaction, which results in formation of new normal-appearing connective tissue throughout the interstices of the sponge. In this paper, rate of collagen formation and degree of acetic acid solubilization of collagen in 7 to 300-day-old connective tissue obtained from male and female albino rats will be presented.

The biological, biochemical and physical chemical characteristics of connective tissue are becoming of increasing interest in medical research because changes which occur with age have been noted in connective tissue(3). Furthermore, changes in connective tissue of the wall of the artery appear to precede the lesion of atherosclerosis(4).

Methods. Adult male and female albino rats were implanted with 3 sponges each along the dorsum(2). The biopsy-connective tissues from 3 male and 3 female rats were removed at 7, 14 and 21 days after implantation and from another group of 3 male rats at 10, 17 and 24 days. The connective tissue biopsies were removed from 5 male and 5 female rats after 100 days and from a male and a female rat after 300 days of tissue growth. After removal of the sponge implants, the tissue biopsy was frozen at -26°C in order to facilitate the removal of the capsule. The sponge-connective tissue was weighed and homogenized in physiological saline (pH 7.4) in a VirTis homogenizer with

a rheostat setting of 50. for 5 minutes. The resulting homogenate was centrifuged at 13,000 rpm at 5°C for one hour to separate the saline-soluble fraction of the tissue from the residue. After decanting the supernatant, the residue was washed once with distilled water at 5°C and centrifuged at 13,000 rpm at 5°C for half an hour. The water wash was discarded. The saline-insoluble residue was hydrolyzed with 6 N HCl in a sealed pyrex tube for 16 hours at 110°C . The nitrogen concentrations of the saline homogenate and the acid hydrolysate of the residue were determined by the Conway microdiffusion technic(5) and converted to protein by the usual factor of 6.25. Hydroxyproline, which was determined by the method of Neuman and Logan(6), was multiplied by the factor of 7.46 to obtain the collagen value. The non-collagenous protein of the residue was obtained by subtracting the collagen value from the total protein value for the residue. One-half molar acetic acid was used for the solubilization of the saline-insoluble protein. The insoluble protein was extracted at 5°C for 3 successive 24-hour periods. After each extraction, the sample was centrifuged at 29,500 rpm at 5°C for 1 hour in a Spinco Model L Ultracentrifuge to obtain the acetic acid-soluble fraction. Protein contents of the acetic acid-soluble and -insoluble fractions were determined. All data were calculated on the basis of weight of the dry sponge implanted.

Results. The content of protein and percentage distribution of the protein of the rat sponge-connective tissue at 7 to 300 days of tissue age are recorded as mean values in Table I. The collagenous protein of the saline-insoluble residue increased rapidly during the first 21 days of growth in both sexes. Beyond 21 days of tissue age, the collagen of the connective tissue from the male rat continued to increase while in the female rat, the collagen content remained relatively constant.

When the collagen content of the newly

TABLE I. Protein of Rat Biopsy-Connective Tissue.

Tissue age (days)	mg/g sponge				% distribution		
	Saline-soluble	Saline-insoluble		Total	Saline-soluble	Saline-insoluble	
		Collagen	Non-collagen			Collagen	Non-collagen
♂ 7	335	11*	173	519	64	2	34
10	418	37	200	655	63	6	31
14	288	77	187	552	56	14	30
17	382	113	224	719	53	15	32
21	321	180	198	699	46	26	28
100	349	386*	195	930	37	42	21
300	666	475	314	1455	46	33	21
♀ 7	409	28*	176	613	65	4	31
14	384	103	218	705	57	15	28
21	349	169	201	719	49	23	28
100	353	198*	248	799	46	28	26
300	400	160	193	753	53	21	26

* $p < 0.05$ when male and female data compared.

forming connective tissue was plotted against the age of the tissue in days on semilogarithmic scale (Fig. 1), there was a linear relationship between collagen content and tissue age during the initial 14-day period in the female tissue and during the 21-day period in the tissue of the male. The slopes and rate constants of the linear portions of the 2 curves were the same.

The effect of acetic acid upon the saline-insoluble protein of the biopsy-connective tissue as the tissue ages is shown in Table II. The acetic acid-solubilized protein increased in amount from 7 to 300 days of tissue age. The increase in the tissue of the female was not as great as in the tissue of the male. However, the percentage of the total saline-insoluble protein which was solubilized by acetic acid decreased with connective tissue age in both sexes, and this reduction in solubilization at 300 days' tissue growth was more marked in the male than in the female (Table

II).

Discussion. Following implantation of the sponge into the rat, a wall of fibroblasts and amorphous material envelops it as a capsule and the interior of the sponge becomes filled with an eosin-staining material. At the 4th to 5th day following sponge implantation, the eosin-staining material coalesces into strands, which are argentophilic. The fibroblasts then move into the sponge and occupy the periphery of the sponge by the 7th day. At the 7th day, hydroxyproline was detected in the connective tissue biopsy which had been freed of its capsule. The presence of this amino acid is indicative of the beginning of the collagen structure.

Work reported from these laboratories indicated an electrophoretic similarity between saline-soluble protein of the tissue and serum protein(7). This soluble fraction of the tissue contained protein components which migrated to the albumin, the alpha, beta and gamma globulin zones of serum run simultaneously. The protein of the saline-soluble fraction is apparently derived from the serum within the first few days following sponge implantation and remains in equilibrium with the serum during the 100 to 300 days of connective tissue development.

The non-collagenous protein does not appear to be an essential part of the collagen structure because it is separated from the collagen of the acetic acid-soluble fraction of the connective tissue as the latter precipitates

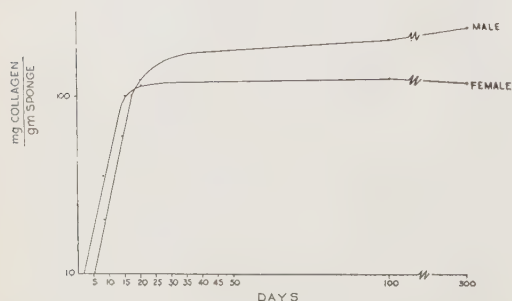


FIG. 1. Rate of development of collagen of rat biopsy-connective tissue.

TABLE II. Saline-Insoluble Protein of Rat Biopsy-Connective Tissue.

Tissue age (days)	Total, mg/g sponge		Acetic acid-soluble, mg/g sponge		% solubilized	
	♂	♀	♂	♀	♂	♀
7	142.2 ± 15.6 (3)*	164.6 ± 44.0 (3)	35.7 ± 7.1	52.0 ± 15.1	24.9 ± 3.2	31.8 ± 4.1
21	398.4 ± 53.1 (6)	373.6 ± 38.2 (9)	63.5 ± 17.3	38.0 ± 8.0	16.2 ± 5.5	10.3 ± 2.0
300	1455.2 (1)	369.4 (1)	153.0	70.0	12.6	19.7

* Figure in parenthesis indicates No. of tissues.

during dialysis. The crystalline-like strands of collagen which then form have the characteristic hydroxyproline concentration of 13.6% and an intrinsic viscosity of 12 to 13. Yet the non-collagenous protein of the acetic acid-soluble fraction is apparently firmly bound to collagen because the acid-soluble material behaves as a monomer during ultracentrifugation at 250,000 G for one hour at 6°C.

Factors which control rate of collagen synthesis are not understood. The fact that the collagen content of the tissue from the female remained constant after 21 days of growth would appear to indicate a slow rate of collagen formation as suggested by studies on incorporation of C¹⁴-labeled glycine in tendons (8). It might also indicate that collagen synthesis rate in the connective tissue of the female equals rate of collagen breakdown. Current research in our laboratories indicates that it is this latter state of equilibrium which probably occurs. The connective tissue from the male continued to increase in collagen content up to 300 days of tissue growth. Apparently in the male rat, rate of collagen synthesis surpasses rate of collagen degradation. Some factor, presumably hormonal, apparently enhances the synthesis and breakdown of collagen in connective tissue from the female rat, and it would appear that this factor is less active in the male rat.

The percentage of scleroprotein solubilized by acetic acid decreased with tissue age, and this finding is similar to that reported by Orekhovich(3) and Nageotte(9) in their studies on the acid extraction of skin. This tissue age difference in the solubility of collagen is thought to be related to the degree of cross-linking of the polypeptide strands.

It is also noted that the 300-day-old tissue from the female rat had a lower collagen content and yet a greater percentage of solubilized collagen than did the tissue of the male. This suggests that not only is the collagen of the female rat metabolically more active than the collagen of the male but that the internal bonding of the collagen obtained from the female rat is more easily disrupted by weak acids than that of the male.

Summary. (1) Collagen was found initially in connective tissue biopsy of male and female rats at 7 days following sponge implantation. (2) Collagen content of connective tissue biopsy increased rapidly in both sexes during the first 14 days of tissue growth. (3) After 21 days of connective tissue development in the female, collagen content remained constant up to 300 days of tissue age while in the male, collagen content continued to increase as the tissue aged. (4) The percentage of total scleroprotein which was solubilized by 0.5 M acetic acid decreased with tissue age and this decrease was greater in the 300-day-old tissue of the male rat than in comparable tissue of the female.

The authors gratefully acknowledge the work of Dr. Maurice Lev, Mt. Sinai Hospital and University of Miami School of Medicine, in preparation of histological sections of the tissue and encouragement and continued interest of Dr. E. Sterling Nichol.

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Effect of Alternating Periods of Underfeeding and *Ad libitum* Feeding on Insulin Sensitivity of Rats.* (23280)

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Prolonged underfeeding (4-6 weeks on 10 g of diet daily) followed by a 24 hr fast causes an increase in the hypoglycemic response to insulin in adult male rats(1). In the present study fasting insulin sensitivity of a group of rats was examined after a period of undernutrition, during a subsequently instituted *ad libitum* feeding regimen, and in the course of a second period of undernutrition.

Materials and methods. Seven male Sprague-Dawley rats, serving as their own controls, were used. The mean initial weight of the group was 281 ± 4 (S.E.) g. All in-

sulin sensitivity determinations were undertaken after a 24 hr fast. One tenth U of insulin (Iletin-Lilly) per kg was injected i.p. Blood sugar levels were ascertained as in our previous study(1). The first insulin sensitivity measurement (Fig. 1, A) was performed 5 weeks after the initiation of a restricted feeding regimen (10 g ground Rockland diet daily), the second (Fig. 1, B) after 3 weeks and the third (Fig. 1, C) after 6 weeks of subsequent *ad libitum* feeding of the same diet. Following the third determination daily food rations were again reduced by 2 g daily from

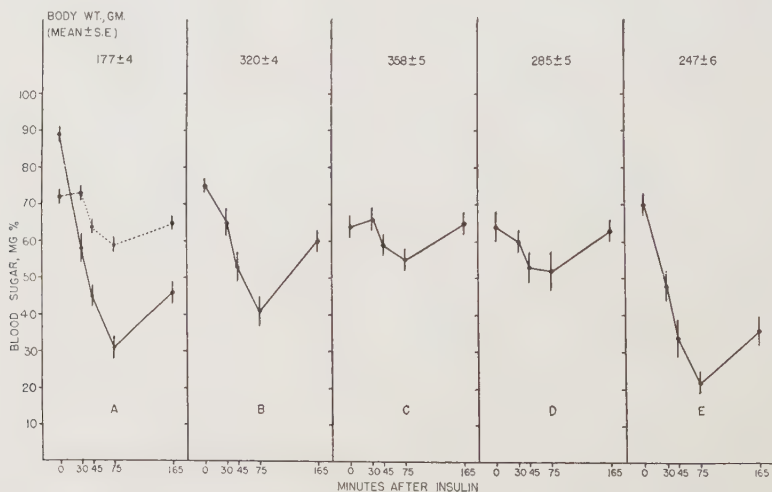


FIG. 1. Blood sugar levels before and after insulin in fasted rats after prolonged undernutrition (A), during *ad libitum* feeding (B and C) and renewed undernutrition (D and E). Details in text. Dotted line: values in 23 *ad libitum* fed-fasted rats of a previous study(1). Vertical lines: stand. errors of the mean.

* Supported by grant, U.S.P.H.S.

18 to 10 g daily, and the next 2 insulin sensitivity measurements were made after the animals had consumed 10 g food/day for 4 weeks (Fig. 1, D) and 9 weeks (Fig. 1, E) respectively.

Results. Prolonged undernutrition led to relatively high fasting blood sugar levels and increased insulin sensitivity (Fig. 1, A). Nutritional rehabilitation was accompanied by progressive lowering of fasting blood sugar titers and gradual return of normal insulin sensitivity (Fig. 1, B and C). A second period of underfeeding at first did not lead to any change (cf. Fig. 1, C and D). However, upon further prolongation of undernutrition marked insulin sensitivity returned, although it was not accompanied by fasting blood sugar levels higher than those found in *ad libitum* fed rats (cf. Fig. 1, A and E).

Discussion. These findings demonstrated that *ad libitum* feeding abolished both the relative hyperglycemia and hypersensitivity to insulin in the fasted state which are characteristic of underfed rats, and that return of normal insulin resistance was not due to

immunity to insulin which had arisen because of its repeated administration, since during a second period of underfeeding excessive insulin sensitivity again developed, although less rapidly than in the course of initial undernutrition. Systematic studies of the influence of age on fasting glycemia and insulin sensitivity and on the effects of underfeeding on these aspects of carbohydrate metabolism remain to be done. However, in our previous study(1) we have not noted any consistent differences in these regards among rats whose weight ranged from 180 to 350 g at the inception of underfeeding or, if the animals were fed *ad libitum*, at the time of determination of insulin sensitivity.

Summary. The characteristic fasting insulin hypersensitivity of chronically underfed rats is gradually abolished by subsequent prolonged *ad libitum* feeding and slowly returns during repeated undernutrition.

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Influence of Adrenergic Blockade and an Amine Oxidase Inhibitor on Reserpine Hypotension. (23281)

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The hypotensive effect of intravenous reserpine in anesthetized dogs requires 2 hours to become significant(1). Recent evidence which indicates that reserpine releases a sympathetic neurohumoral agent by Holzbauer and Vogt(2) and Maxwell *et al.*(3) suggests that this agent might be acting to mask a rapid onset of reserpine action. We have tested this hypothesis by determining the effect of adrenergic blockade and amine oxidase inhibition on the onset of reserpine hypotension. The following is a report of our findings.

Methods. Twenty-five dogs anesthetized with 30 mg/kg sodium pentobarbital intraperitoneally and a continuous intravenous in-

fusion of 0.1 mg/kg/minute of sodium pentobarbital were used. Femoral arterial pressures were recorded with the Anderson glass capsule manometer(4). All drugs were administered intravenously. Phentolamine HCl was administered over a 15-30 minute period to minimize its hypotensive effect. Observations on hypotension produced by reserpine were limited to the first half hour after reserpine administration. Choline p-tolyl ether bromide, an amine oxidase inhibitor first reported by Brown and Hey(5) was employed in these experiments.

Results. *Reserpine phosphate on blood pressure.* In 6 dogs 0.5 mg/kg intravenous reserpine phosphate produced no significant

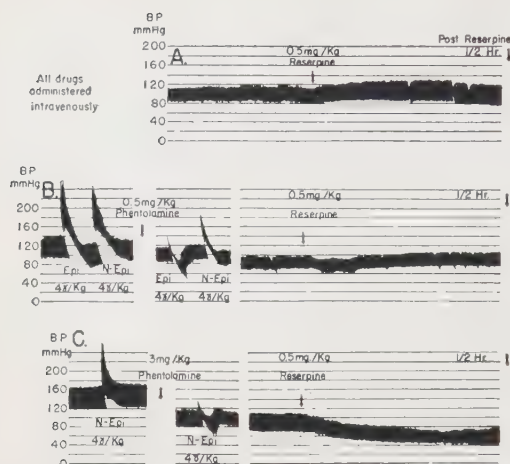


FIG. 1. Effect of adrenergic blockade on blood pressure response to reserpine phosphate.

change in blood pressure. Mean pressures varied from +5 to -10 mm Hg (-4 mm Hg average) over a half-hour period (Fig. 1-a).

Phentolamine (0.5 mg/kg) plus reserpine phosphate on blood pressure. Five-tenths mg/kg phentolamine produced a reversal of epinephrine pressor spikes and only a moderate reduction in nor-epinephrine pressor spikes in 4 dogs (Fig. 1-b). Subsequent administration of 0.5 mg/kg of reserpine phosphate resulted in insignificant variations in blood pressure ranging from -5 to +10 mm Hg (+2 mm Hg avg) over a half-hour period (Fig. 1-b).

Phentolamine (3 mg/kg) plus reserpine phosphate on blood pressure. Three mg/kg of phentolamine produced a very marked reduction of pressor response to nor-epinephrine in 7 dogs (Fig. 1-c). Subsequent administration of 0.5 mg/kg of reserpine phosphate produced changes in blood pressure ranging from -15 to -40 mm Hg (-30 mm Hg avg) during the first half-hour (Fig. 1-c). These reserpine-induced decreases in pressure could be demonstrated after phentolamine pretreatment which had lowered arterial pressures to levels ranging from 50 to 100 mm Hg.

Amine oxidase inhibitor plus reserpine phosphate. Ten mg/kg choline p-tolyl ether bromide were administered to 8 dogs. When the transient pressor spikes produced by this compound subsided, 1 mg/kg reserpine phosphate was injected. Reserpine phosphate ad-

ministration resulted in pressor responses in 7 dogs which ranged from +10 to +40 mm Hg (avg +20 mm Hg) and lasted throughout a half-hour period (Fig. 2-a). In the 8th dog there was a blood pressure decline of 30 mm Hg after reserpine administration.

The effect of 10 mg/kg of choline p-tolyl ether upon the pressor responses to serotonin, epinephrine and nor-epinephrine was determined (Fig. 2-b). Responses to epinephrine and nor-epinephrine were potentiated by choline p-tolyl ether. The response to serotonin was prolonged but not dramatically enhanced.

Discussion. It is concluded from this work that the reduction in blood pressure produced by reserpine after phentolamine pretreatment is not a reflection of the epinephrine-reversal phenomenon. Concomitantly it is concluded that the vasoactive sympathomimetic humoral agent released by reserpine that is of greatest importance in sustaining blood pressure is not epinephrine but is most likely nor-epinephrine; and that this released substance masks an immediate moderate hypotensive effect of reserpine.

Pressor responses produced by the amine oxidase inhibitor plus reserpine further support the hypothesis of a rapid release of a sympathetic humoral factor by reserpine. The more dramatic potentiation of nor-epinephrine and epinephrine as compared with serotonin would suggest that serotonin plays only a minor role in the pressor response. Chessin *et al.*(6) have reported that iproniazid pretreatment will also cause reserpine to produce pressor responses.

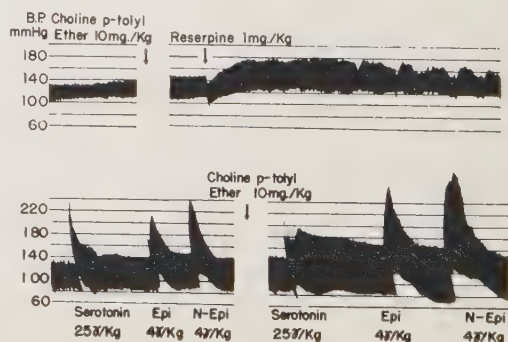


FIG. 2. Effect of amine oxidase inhibitor on blood pressure response to reserpine phosphate and pressor agents.

Summary. In proper doses administration of reserpine phosphate subsequent to adrenergic blockade with phentolamine will produce a more rapid decline in blood pressure than is commonly obtained by administration of reserpine alone. After pretreatment with amine oxidase inhibitor, choline p-tolyl ether, reserpine will produce pressor responses. These data are interpreted as supporting the hypothesis that reserpine releases sympathomimetic humoral agents which mask the early onset of the cardiovascular actions commonly attributed to reserpine.

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Effect of Increased Diphosphopyridine Nucleotide Levels on Rate of Ethanol Metabolism in the Mouse. (23282)

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The rate-limiting reaction in total oxidation of ethanol to CO_2 is the first step in the sequence of oxidation of ethanol to acetaldehyde(1). Three separate entities, ethyl alcohol, diphosphopyridine nucleotide,* and the enzyme alcohol dehydrogenase, participate in this reaction. It is generally agreed that the rate curve for disappearance of ethanol from blood follows a straight line rather than an exponential curve, except at low concentrations of blood alcohol(2). The enzyme, therefore is soon saturated with its ethanol substrate, and the availability of the alcohol does not appear to be a physiologically significant rate-limiting factor in its oxidation. This study was initiated in an attempt to determine whether the DPN concentration or amount of alcohol dehydrogenase present in liver may be the controlling factor in the disappearance of ethanol from the body.

Kaplan and coworkers(3) have induced DPN levels 10 times that of normal in the mouse liver by injection of nicotinamide 8-12 hours previously. Mice preinjected in this manner have been used in this work to study

possible effects of the heightened DPN level on rate of ethanol oxidation. Both *in vivo* and *in vitro* studies have been performed.

Methods. *In vivo experiments.* Blood alcohol concentration was determined by the method of Newman and Newman(4). Adult mice of the C57 BL strain maintained on Purina Laboratory Chow were used. Six hours before administration of alcohol, the mice were injected intraperitoneally with 500 mg/kg nicotinamide in a solution containing 25 mg/ml. This time interval was chosen so that during the latter part of the 5 hours after alcohol administration, the DPN would be at optimum level in the liver as reported by Kaplan *et al.*(3). The ethyl alcohol was injected in 20% aqueous solution w/v in a single intraperitoneal injection at 4 g/kg. Alcohol injections were made to both mice preinjected with nicotinamide and to their untreated controls. At hourly intervals for 5 hours after alcohol injection, nicotinamide treated animals and controls were anaesthetized with chloroform and blood withdrawn from the heart which resulted in their death.

In vitro experiments. Slices. Webster Swiss mice were injected with 500 mg/kg nico-

*Diphosphopyridine nucleotide will be abbreviated to DPN

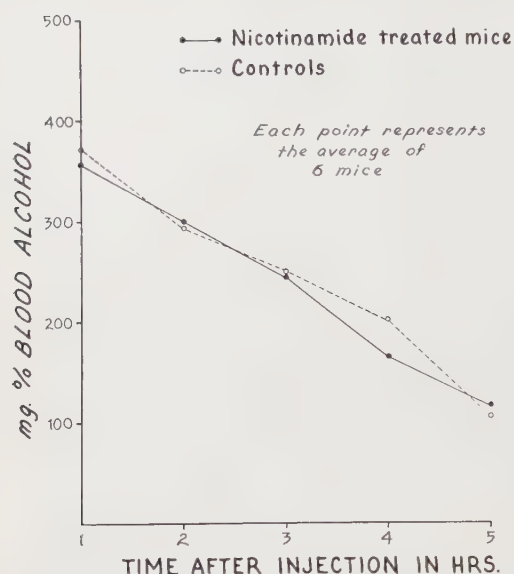


FIG. 1. Fall in blood alcohol levels after ethanol inj. in mice pre-inj. with nicotinamide and their controls.

tinamide 8 hours before killing. These and untreated mice were killed by decapitation, the livers quickly excised and sliced to a thickness of approximately 0.5 mm by a Stadie-Riggs microtome. The slices were immersed in 25 ml Erlenmeyer flask which contained 3.5 ml 0.1 M phosphate buffer at pH 7.5 and 4.5 mg ethyl alcohol. About 400 mg of slices were used/flask. Air above the mixture was replaced with oxygen, then the flask was stoppered and allowed to incubate in Dubnoff Metabolic Shaker at 37°C. After 3 hours, 1 ml of 50% trichloroacetic acid was added to stop the reaction. Appropriate controls contained trichloroacetic acid in the original incubation medium. The contents of the flasks were centrifuged and the supernatant fluid was analyzed for alcohol by the enzymatic method of Marshall and Fritz(2). *Homogenates.* The mice were preinjected as for the slice experiments. Homogenates were made from normal and pretreated livers by homogenizing the liver with 2 parts of 0.1 M phosphate buffer pH 7.5 which contained 50 mg nicotinamide/ml to minimize nucleotide destruction. One ml of this homogenate was added to the incubation medium which contained 3 ml 0.1 M phosphate buffer at pH 7.5 and 4.5 mg ethyl alcohol. The flasks were

TABLE I. Total Ethanol Metabolized by Liver Slices from Normal Mice and from Mice Pre-injected with Nicotinamide. Complete system contains 3.5 ml 0.1 M phosphate buffer at pH 7.5, 4.5 mg ethanol, and 400 mg liver slices incubated for 3 hr at 37°C under oxygen.

	Ethanol metabolized (mg)	
	Control	Pre-inj.
Exp. 1	1.35	1.15
2	1.47	1.38

gassed, incubated, and analyzed as described for slice experiments.

Results. As shown in Fig. 1, the nicotinamide-injected animals did not metabolize more alcohol than did the control animals in the 5 hour period tested. There was wide variation among individuals, but except for the 4 hour point, the average blood alcohol levels of the 2 groups followed each other closely. The total fall in blood alcohol concentration in the nicotinamide-treated animals was 240 mg %, while in controls it was 264 mg %.

In the slice experiments the results are similar, as shown in Table I. High levels of DPN in the liver did not increase the rate of alcohol metabolism.

The homogenate experiments show a definite increase in total alcohol metabolized (Table II) by the preinjected animals. Undoubtedly, in spite of the large amounts of nicotinamide added during the homogenization process, the rapid destruction of DPN which takes place in homogenates by nucleotide phosphatases has made this coenzyme the limiting factor. These data serve to confirm the finding of Kaplan and coworkers(3) that a definitely higher level of functioning DPN is induced in the animals pretreated with nicotinamide.

Discussion. Our results show that, in the

TABLE II. Total Ethanol Metabolized by Homogenates of Livers of Normal Mice and Mice Pre-injected with Nicotinamide. Complete system contains 3.0 ml 0.1 M phosphate buffer at pH 7.5, 4.5 mg ethanol, and 1 ml liver homogenate. The mixture was incubated under oxygen for 3 hr at 37°C.

	Ethanol metabolized (mg)	
	Control	Pre-inj.
Exp. 1	1.53	2.09
2	1.29	2.07
3	1.01	2.06

well-fed mouse, the physiological level of DPN is sufficient to maintain a maximum rate of conversion of ethanol to acetaldehyde. The alcohol dehydrogenase appears to be fully saturated with DPN as well as with ethanol. The enzyme content in the mouse liver therefore appears to control the rate of the first step of alcohol oxidation, and since this reaction limits the rate of subsequent oxidations, the amount of enzyme present must be the limiting factor in the whole sequence. If this is the case, it is difficult to understand how various metabolites are able to increase the rate of alcohol disappearance in the well-fed animal, as is frequently reported. Vitale *et al.*(5) have emphasized the importance of adequate diet for animals used in research on alcohol metabolism, and studies are in progress in this laboratory to determine the effect of fasting on DPN and alcohol dehydrogenase levels.

Summary. Mice, in which a high level of DPN was induced by injection of nicotinamide, metabolized ethanol no more rapidly than untreated controls. This is interpreted to mean that in an animal receiving adequate niacin in its diet, availability of DPN does not limit the disappearance of ethyl alcohol from the body.

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Ice as a Mechanical Factor in Death of Spermatozoa on Freeze-Thawing.* (23283)

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A consideration of ice formation has figured prominently in the elucidation of theories concerning the lethal consequences of cooling protoplasm below its freezing temperature. Physical destruction by ice in the medium surrounding the cell, within the cell, or at both sites, has been proposed as a salient causative factor in death on freezing and thawing(1,2). This proposition has provoked the suggestion(3) and application(4) of a theory of survival based upon vitrification by rapid cooling. At the time glycerol was introduced as a protective agent which permitted revival of spermatozoa after cooling to -79°C , mechanical injury by ice and the advantage of vitrification were pertinent considerations in attempts at preservation of cells by freezing. In fact, the term vitrification

was used in lieu of freezing in the very paper which reported the protective action of glycerol(5). Further, the results of direct microscopical observations on freezing of fowl semen were said to indicate that glycerol modifies ice formation and dissolution in the medium so that damage due to pressure and other mechanical effects is reduced(6). Recent work(7), however, strongly suggests that damage on freezing and thawing spermatozoa of the bull, rabbit, fowl, and herring, can be attributed to increased salt concentration. Although glycerol has been shown to modify ice formation while favoring survival, on freezing semen of bull and man, no evidence has been presented for physical destruction by ice in the absence of this substance(8,9).

The present report concerns a further study of the possible role of ice as a mechanical factor in death. The object was to ascertain whether or not there is a relationship between

* This investigation was supported, in part, by Research Grant from N.I.H., Public Health Service.

physical strength or weakness of bull spermatozoa, and the susceptibility of these cells to freezing and thawing. Bull spermatozoa are particularly well suited for such an investigation since there is variation in freezability of samples from different bulls, and the number of motile cells provides a readily observable criterion of survival.

Materials and methods. In an attempt to correlate the physical strength of bacteria with their susceptibility to repeated cycles of freeze-thawing, Harrison and Cerroni(10) employed a Mickle tissue disintegrator to induce mechanical injury. This suggested the use of the disintegrator in the present investigation. It was necessary to adjust the apparatus and period of agitation so as to cause immobilization of only some of the spermatozoa in a sample. The extent of immobilization was considered a measure of sensitivity or susceptibility to mechanical injury on shaking. Values of motility were obtained through duplicate readings made on each sample, in a series of different samples, without prior knowledge of sample identity. In our study, 92 samples from 38 different bulls were evaluated. Each was diluted and processed in stepwise fashion so as to contain 15 million spermatozoa/ml in 20% egg yolk (by volume) and 7% glycerol (by volume) in aqueous 2% sodium citrate dihydrate(9). Portions of the sample at 5°C were then removed, one for shaking and the other for freeze-thawing. A 4 ml portion was placed in the disintegrator vial and agitated at maximum setting for 5 seconds, at which time a fraction was removed and placed in a 3 ml test tube at 5°C. One ml portions of the other part were frozen to -95°C in 2 ml sealed glass ampuls and thawed in bath at 5°C. Identical conditions for shaking and freeze-thawing were maintained for all samples compared. Motility observations were made, during one period, on a series of samples which were taken 1) shortly after glycerolation (pretreatment or control), 2) following removal from the disintegrator, and 3) subsequent to thawing from -95°C. Percent survival was computed by dividing percent of motile spermatozoa after treatment by the percent motile before treatment and multiplying by 100.

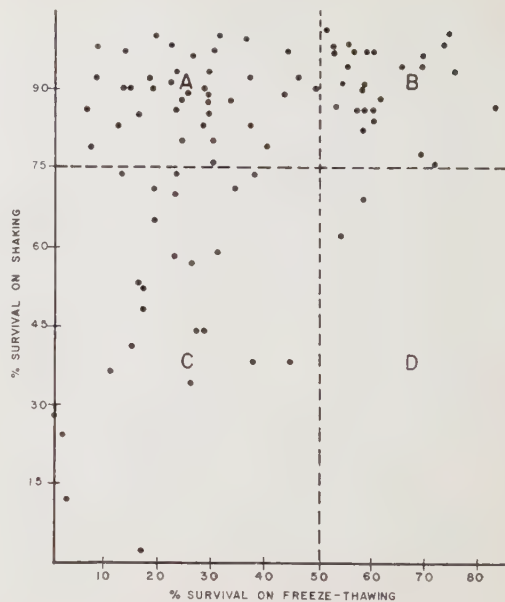


FIG. 1. Scatter diagram showing relationship between survival of spermatozoa on shaking in a disintegrator and on freeze-thawing. A dot represents such survival results for each semen sample. Broken lines separate diagram into areas of high and low survival (A-D), as described in text.

Results. Results are summarized in Fig. 1. Experience and survey of the data suggested the designation of over 75% on shaking and over 50% on freeze-thawing as high survival values, while figures below these were considered low. The data indicate the following:

1) There is a very poor correlation between survival on shaking and that realized on freeze-thawing. If correlation were good, most dots would be located in areas B and C, reflecting a direct relationship between survival subsequent to treatments. This was not the case, as more than 43% of the dots fell outside these areas.

2) There is a very poor correlation between high survival on shaking and results of freeze-thawing. If correlation were good, most values of 75% and above, on shaking, would be located in area B. However, only 40% of these dots fall in this area with remaining 60% in area A.

3) There is a very good correlation between low survival on shaking and low survival on freeze-thawing. Ninety-two percent of samples sensitive to shaking were also sensitive to freeze-thawing, as shown by the lop-

sided concentration of dots in areas C as compared with D.

4) High survival on freeze-thawing was observed, with 2 exceptions out of 29, only in those samples which showed high survival on shaking, as seen in areas B and D.

Discussion. In view of present meager knowledge of the mechanisms in life and death at low temperatures, the interpretation of these findings could easily be misleading. The conclusion that physical destruction by ice is eliminated as a possible factor in death on freeze-thawing bull spermatozoa is tempting, and might derive support from reports of Lovelock(7) and Sherman(9). However, it involves the assumption that agitation in a tissue disintegrator produces mechanical injury to a cell similar to that realized on freeze-thawing. But possibly the damage on agitation is of a chemical nature. The number of motile cells in bull semen was reported to decrease on shaking during storage, due to production of hydrogen peroxide, the deleterious action of which is obviated in the presence of catalase(11). Our own experiments with these substances under conditions of the present investigation show that hydrogen peroxide is not the responsible factor. Nevertheless, this does not rule out the possibility that agitation in a disintegrator produces chemical changes, such as oxidations, which induce immobilization. Assuming that the effect of shaking is solely mechanical and is similar to that realized through compression and crushing by extracellular ice(10), there still remains the possibility of damage by ice crystallization within the cell. This would then demand further consideration.

Based upon our results and existing knowledge, it appears that ice is not a mechanical factor in death of bull spermatozoa on freeze-thawing. A more positive conclusion cannot be expected until more complete information on low temperature phenomena becomes available.

Most likely, sensitivity to shaking merely reflects the sensitivity of bull spermatozoa to one of various damaging environmental factors, including altered salt concentration, pH, etc. Relevant to the other data, the fact that 92% of the samples sensitive to shaking were also sensitive to freeze-thawing, not only supports the idea of environmental insult, but also suggests a method for identifying and screening out some of the semen samples which will show low survival on freeze-thawing.

Summary. An attempt was made to ascertain whether or not mechanical injury by ice is a factor in death of bull spermatozoa on freeze-thawing. A tissue disintegrator was employed to produce mechanical injury by shaking. Results of an attempted correlation between survival on shaking and on freeze-thawing suggest that death on freeze-thawing is not caused by mechanical injury by ice.

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Effect of High Speed Centrifugation on Distribution of Serum Cholesterol. (23284)

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The physical state in which cholesterol occurs in the plasma has received a great deal of attention during the last few years, primarily on account of its possible relationship to development of atherosclerosis. We have been interested in a fraction of the total serum cholesterol which is removed when lyophilized serum is extracted for 3 hours with cold anhydrous chloroform[‡] according to a previously described procedure(1). We consider the normal concentration of this "readily extractable cholesterol" (R.E.C.) to be 40 mg or less/100 ml serum since only 5 of 461 male subjects between 17-25 years of age showed values higher than this(2). An elevated concentration was found in 35% of 259 patients who had suffered a myocardial infarction(3). Patients with arteriosclerotic heart disease also tended to have an abnormally high concentration. A very high percentage of the total serum cholesterol of patients with nephrosis was associated with this fraction.

Methods. To obtain information on distribution of this fraction in serum, values for the R.E.C. of serum and of the subnatant portion of the same serum following centrifugation at 20,000 g for 2 hours have been compared. Centrifugation was carried out in cold room about 6°C, using plastic tubes and an angle centrifuge. Following centrifugation, each tube was removed separately, using the utmost care to prevent mixing. The tube was rested on the centrifuge for support and a 0.5 ml pipette, the top closed with a finger, intro-

duced to bottom of tube. After removal of the sample, the tip was wiped with filter paper before adjustment to the mark. The subnatant serum was transferred to a one-half ounce bottle and lyophilized in the usual manner(1). Although no dark field examination of the subnatant fluid was carried out we feel that most, if not all, of the chylomicrons would have risen to the surface during centrifugation but that lipomicrons would not rise. Since the cholesterol present in both chylomicrons and lipomicrons is probably extracted by cold chloroform(4), we assume that the difference between the amount of R.E.C. in the uncentrifuged serum and in the subnatant fluid after centrifugation represents cholesterol which was associated with chylomicrons and rose to the surface during centrifugation. Although the chief purpose of the present study was to determine the relationship, if any, which might exist between the R.E.C. of the serum and amount of cholesterol which rose to the surface during centrifugation, emphasis was laid on selecting subjects diagnosed as having atherosclerosis or as having disease conditions which predispose to atherosclerosis. About two-thirds of the cases had had a myocardial infarction at some previous time or had arteriosclerotic heart disease. The remaining subjects represented a variety of conditions such as diabetes, xanthlasma, xanthomatosis, idiopathic hyperlipemia, and nephrosis, as well as a number of apparently normal individuals.

Results. The general pattern of results is shown in Table I. It will be seen that when the R.E.C. concentration was within normal limits (below 41 mg %) only a small percentage of total serum cholesterol rose to the surface during centrifugation. Sera with elevated R.E.C. values usually showed a greater percentage of the total cholesterol rising to the surface. This was especially marked in sera in which the R.E.C. concentration was

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‡ To assure uniformity we use "Baker Analyzed" reagent grade chloroform. About 10 g of anhydrous sodium sulfate is added to each 5 lb bottle and the bottles shaken off and on for several days before using.

TABLE I. Readily Extractable Cholesterol Concentration of Serum and of the Supernatant Fluid after Centrifugation at 20,000 g.

No. of tests	Total cholesterol Serum, avg	Readily extractable cholesterol				% R.E.C. rising
		Serum Range	Serum Avg	Subnatant, avg	Serum minus subnatant, avg	
		mg %				
75	245	<41	29	22	7 ± .5*	24
48	303	41-70	56	32	24 ± 1.8	43
31	327	71-150	118	40	78 ± 4.6	66
21	324	151-250	204	46	158 ± 5.2	77
27	545	>250	455	107	348 ± 42.0	76

* Stand. error.

above 70 mg %. On the whole the higher the R.E.C. value the greater the proportion of total serum cholesterol which rose to the surface during centrifugation. One case with idiopathic hyperlipemia with a total serum cholesterol of more than 1,000 mg % showed over 90% of total serum cholesterol rising to the surface. The supernatant layer in this case was solid at the end of the centrifugation.

A few subjects with a variety of clinical conditions showed quite a different picture from this general pattern. These showed either no cholesterol or only very little of it rising to the surface during centrifugation in spite of the fact that the R.E.C. was markedly elevated. Five of these subjects had

nephrosis, 3 had diabetes of long duration, 2 had myxedema secondary to radio-iodine administration, one a myocardial infarction superimposed on a previous cerebrovascular accident, and one apparently was a normal subject. These results are shown in Table II.

We have no satisfactory explanation for the difference in serum cholesterol distribution of these subjects when compared with the others who showed markedly elevated R.E.C. concentrations. The fact that the sera of 5 of the 6 nephrotic patients showed very little cholesterol rising to the surface during the centrifugation is difficult to explain. Albrink, Mann, and Peters(5) found a large percentage of the total serum cholesterol of a 3-year-

TABLE II. Difference between the Readily Extractable Cholesterol Concentration of the Serum and Supernatant Fluid in Sera Which Did Not Conform to the Usual Pattern, in 13 Patients.

Age, yr	Sex	Total serum cholesterol, mg %	Readily extractable cholesterol, mg %			Diagnosis and remarks
			Serum	Subnatant	Serum minus subnatant	
21	♂	1138	1130	986	144	Nephrosis with edema
		1166	1030	1041	nil	4 days later
40	♂	509	465	463	nil	Nephrosis with edema
		618	589	589	nil	25 days later
27	♀	652	571	573	nil	Nephrosis, 17 days postpartum
3	♂	478	443	440	nil	Nephrosis
4	♀	852	822	500	322	"
40	♀	467	411	327	84	Diabetes for 15 yr
		465	413	315	98	6 days later
53	♀	509	456	456	nil	Diabetes for 30 yr
69	♀	350	257	204	53	" " 26 yr
37	♂	545	495	500	nil	Mitral stenosis with edema at intervals for 2 yr
56	♀	521	448	404	44	Myocardial infarction superimposed on previous cerebrovascular accident
50	♂	691	583	530	53	Myxedema from radio-iodine
72	♀	618	488	500	nil	<i>Idem</i>
54	♀	386	282	224	58	Apparently normal subject

old nephrotic child, rising to the surface when the serum was centrifuged at 20,000 g for 1 hour. All patients studied by us were under some form of treatment when blood samples were collected, but since the kind of treatment varied it is doubtful whether this can explain why the cholesterol distribution in these subjects was so different from that seen in the majority of the cases. Reference should be made to the fact that in sera of nephrotic subjects who showed a small amount of cholesterol rising to the surface during centrifugation, the degree of lipemia was definitely less than we are accustomed to see in nephrotic patients. Serum phospholipid phosphorus was determined in a number of cases but we were unable to find any definite correlation between these values and the R.E.C. of either the serum or subnatant fluid.

It is, of course, premature to suggest any diagnostic value to the fractionation procedure, but it is interesting that the sera of many patients with myocardial infarction contained significant amounts of cholesterol associated with chylomicrons and rose to the surface during centrifugation. This may be considered as supportive evidence for the colloidal-macromolecular theory of the pathogenesis of atherosclerosis, especially as stated by Heuper(6) and Moreton(7). The results on the nephrotic sera, however, would seem to throw some doubt on this but it is possible that a satisfactory explanation for the apparent discrepancy can be obtained.

Summary. As a general rule a fairly large percentage of total serum cholesterol rose to the surface when sera containing markedly elevated amounts of "readily extractable cholesterol" were centrifuged at 20,000 g for

2 hours. Sera having a normal "readily extractable cholesterol" concentration showed very little of the cholesterol rising to the surface under the same conditions. A few subjects, including most nephrotic patients studied, differed markedly from this pattern. In these cases, only a small amount of cholesterol rose to the surface during centrifugation in spite of the fact that the sera had markedly elevated "readily extractable cholesterol" concentrations. It is suggested that the cholesterol which rose to the surface is cholesterol which is associated with chylomicrons, while that which did not rise but which is a part of the "readily extractable cholesterol" is cholesterol present in lipomicrons.

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Metabolism of C^{14} Labelled Beta-Carotene in the Rat.* (23285)

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Our previous studies on the metabolism of C^{14} labelled beta-carotene have indicated that in the rat it is largely converted to a non-saponifiable substance or substances(1). At that time it was not possible to determine if all radioactivity of the non-saponifiable material resided in vit. A because no method was available for the isolation and purification of vit. A from this fraction. Since then, a method has been developed for isolation and purification of vit. A from the non-saponifiable extracts of liver tissue(2). With this technic it appeared worthwhile to continue our investigation on the metabolism of beta-carotene.

Method. Our previous commercial source of radioactive carotene was not available so it was necessary to develop a technic to produce it. Such carotene was produced by growing *Phycomyces blakesleeanus* in a suitable C^{14} labelled acetate medium.[†] Two samples of labelled C^{14} carotene were prepared. Sample No. 1 contained 1010 μg of beta-carotene with an $E_{1\text{ cm}}^{1\%}$ of 2430 $\frac{1}{2}$ and a total radioactivity of 65,000 cpm. Sample No. 2 contained 1120 μg of beta-carotene with an $E_{1\text{ cm}}^{1\%}$ of 2470 and a total radioactivity of 35,000 cpm. Each sample was prepared and fed to rats numbered 1 and 2 respectively, as previously described(1). Immediately after feeding, rats were placed in a metabolism chamber without food and water. The CO_2 was collected according to procedure of Skipper *et al.*(3). Both rats were sacrificed 24 hours after feeding the carotene. The non-saponifiable material was extracted from the liver and from the total extrahepatic tissue according to the method of Hjarde(4). The tissue extracts were freed from carotene by chromatographic procedures of Glover *et*

al.(5). The carotene-free non-saponifiable extracts are referred to as CFNS fractions. After non-saponifiable material was extracted, the fatty acids were removed from the saponified extracts by acidifying them with 2N HCl, extracting with petroleum ether, and washing the latter extracts with distilled water. These washings were combined with the remainder of the saponified extracts and labelled "water soluble" fraction. The digitonin-precipitable sterols were isolated from the CFNS fraction according to the procedure of Kelsey(6). Vit. A was isolated from liver CFNS material by the procedure of Powell *et al.*(2). The water soluble fraction was oxidized and the CO_2 collected as described by Skipper *et al.*(3). Radioactivity of the various fractions was measured in a Tracerlab windowless gas flow counter and corrected for background. All CO_2 collected was counted in the form of BaCO_3 and corrected for self absorption. Vit. A content of various CFNS fractions was determined according to technic of Glover *et al.*(7). Due to technical difficulties, it was not possible to purify and count the vit. A in total extrahepatic tissues. Extinction of petroleum ether solutions was measured at 450 $\text{m}\mu$ and, accepting the $E_{1\text{ cm}}^{1\%}$ for beta-carotene as 2500, the amount of carotene was calculated.

Results. From Table I it may be noted that the material in feces, urine, and intestinal contents contained approximately 50% of the total radioactive carbon fed. The total non-saponifiable material from these sources amounted to an average of 30% of the dose fed. Therefore, about 70% of the carotene was assumed to be absorbed. The carotene content of the non-saponifiable fraction from feces of rat No. 1 was 340 μg and from rat No. 2 was 360 μg . The fatty acids from feces, urine, and intestinal contents accounted for about 20% of the dose fed.

Table I summarizes the distribution of C^{14} from the labelled carotene in the tissue and

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[†] Unpublished.

[‡] These "E values" were measured in a light petroleum ether (b.p. 30-40°C) solution at 450 $\text{m}\mu$.

TABLE I. Distribution of C¹⁴ in Rats after Oral Administration of C¹⁴ Labelled β -Carotene.

	Total c.p.m. in sample		% of dose absorbed*	
	Rat 1	Rat 2	Rat 1	Rat 2
Fed	65,000	35,000		
Feces, urine, and intestinal contents	35,000	16,000		
Non-saponifiable	21,000	10,000		
Fatty acids	14,000	6,000		
H ₂ O soluble	—	—		
Total extrahepatic tissue	13,200	10,850	44.0	57.1
CFNS	9,700	7,050	32.3	37.1
Fatty	3,500	3,800	11.7	20.0
H ₂ O soluble	—	—	—	—
Total liver	1,425	872	4.8	4.6
CFNS	765	599	2.6	3.1
Vit. A	425	310	1.4	1.6
Sterols	60	39	.2	.2
Rest	280	250	.9	1.3
Fatty acids	510	173	1.7	.9
H ₂ O soluble	150	100	.5	.5
Expired CO ₂	1,420	1,020	4.7	5.4
Recovered	51,045	28,742		
Non-saponifiable	31,465	17,649		
Fatty acids	18,010	9,973		
H ₂ O soluble	150	100		
CO ₂	1,420	1,020		
Unaccounted for	13,955	6,258		

* Absorbed dose = Administered dose - (amt in feces, urine, and intestinal contents).

fractions examined. In both rats the highest amount of radioactivity resided in the CFNS fraction of extrahepatic tissue. Some activity was present also in the fatty acid fractions. The only "water soluble" fraction found to contain radioactivity was from the liver. About 1.5% of the absorbed C¹⁴ was found in liver vit. A. The combined activity in the CFNS and fatty acid fraction from extrahepatic and liver tissue accounted for an average of about 55% of the absorbed C¹⁴. Approximately 5% of the absorbed C¹⁴ was recovered in the expired CO₂. Thus, about 60% of the absorbed C¹⁴ was accounted for. The average recovery for the total dose fed was about 80%.

The distribution of C¹⁴ in the 3 different fractions of the CFNS material from liver is such that vit. A composes 50% of the total activity of the fraction, the digitonin-precipitable sterols are only slightly active, the remaining non-saponifiable fraction is about as

active as the vit. A fraction. The 2 latter fractions were examined and found to contain no vit. A according to accepted colorimetric or spectrophotometric technics.

Each rat liver contained approximately 3000 μ g of vit. A, and the extrahepatic tissue approximately 150 μ g of the vitamin.

Discussion. It is evident that C¹⁴ from rats fed labelled beta-carotene enters into compounds other than vit. A. These findings support the suggestion of Glover and Redfearn (7) and others (8) that carotene is not entirely converted into vit. A and that it might be directed into other metabolic pathways.

It is interesting to note that considerable radioactivity existed in the fatty acid fraction from feces, urine, and intestinal contents, but no detectable activity was found in the water-soluble fraction from this material. Also, it is of interest that of all the animal tissue, the only water soluble fraction that contained radioactivity was from liver tissue.

The presence of only about 5% of the absorbed C¹⁴ in expired CO₂ is much lower than Laughland (9) reported for the chick and rat. Failure to account for a large amount of C¹⁴ from absorbed carotene is in disagreement with our earlier findings where almost 100% of absorbed C¹⁴ was accounted for in the CFNS fraction. Two explanations seem plausible. One is that the position of labelling of the C¹⁴ in the carotene molecule may have been different in the 2 sources of carotene. The source of carotene in our earlier investigation was from algae which was supposedly uniformly labelled, and in the present study, from fungi where the type of labelling is unknown. Secondly, it is possible that since the animals used contained larger amounts of vit. A than the previous ones, carotene was not needed to supply the animal's need for vit. A, and hence it was directed into secondary metabolic pathways. Studies seeking support for both of these suggestions are now under investigation.

Summary. Feeding of C¹⁴ labelled carotene resulted in the deposition of approximately 1.5% of absorbed C¹⁴ into liver vit. A. In the total animal an average of about 15% of the absorbed C¹⁴ went into fatty acids, and 40% into non-saponifiable material. An av-

erage of about 5% of the absorbed C^{14} was found in the expired CO_2 . Approximately 40% of the absorbed C^{14} remains to be accounted for.

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Hypoglycemic Effect of D-Ribose in Man. (23286)

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Ribose 5-phosphate occupies an important position as an intermediate in the oxidative or pentose phosphate pathway of glucose metabolism. In both plant and animal tissues ribose 5-phosphate forms fructose 6-phosphate(1) which may be readily converted to glucose. Recently Agranoff and Brady(2) have isolated an enzyme from mammalian liver capable of phosphorylating D-ribose to form ribose 5-phosphate, thus permitting ribose to enter into this pathway. The conversion of ribose to glucose has been demonstrated by Katz *et al.*(3) who found that ribose 1- C^{14} incubated with rat liver slices yielded glucose labeled in a pattern which agreed with predictions from known reactions. Intravenous infusion of known glycogenic sugars such as D-galactose(4) and D-fructose(5,6) have been shown to cause an increase in blood glucose levels in normal man. Occurrence of increases in blood glucose levels after infusion of the pentoses D-xylose and L-arabinose has been reported from this laboratory although no effect was observed after D-lyxose and D-arabinose administration(7). Bruck and Rapoport(8) have reported that D-galactose

infusions cause a lowering of blood glucose levels in galactosemic individuals. Except for a single observation that infusion of D-fructose may have a glucose lowering effect (9) no sugars have been observed to cause decreases in blood glucose levels following infusion into normal man. This paper reports that following infusion of D-ribose into normal man, decreases in blood glucose levels occur despite the fact that this sugar is susceptible to conversion to glucose via known metabolic pathways.

Methods. The D-ribose used was obtained from the Pfanstiehl Co., Waukegan, Ill. By the criteria of optical rotation and paper chromatography, the sugar was authentic D-ribose. Fifteen infusions of D-ribose were performed in 5 fasting normal males, (ages 18-21 yrs.) 3 diabetics (ages 23, 31 and 53 yrs.) whose last insulin dose (NPH + crystalline) was given 24 hours prior to study and one 48 yr. old subject with liver disease proved by liver biopsy. D-ribose was autoclaved as a 7.5% solution and found sterile and pyrogen-free prior to use. In 13 studies, 10 to 20 g of D-ribose were infused intravenously over 10-25 minutes. Blood specimens were obtained from an indwelling plas-

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TABLE I. Observations on Glucose and Ribose Levels in Blood after Ribose Infusion.*

Subjects	Fasting blood glucose, mg %	Min blood glucose, mg %	Time to min glucose after start of in- fusion, min.	Blood ribose at time of min glucose, mg %
<i>Normals</i>				
JD	91	59	75	7
"	63	37	56	19
LC	71	30	65	9
WY	52	39	89	10
WB	78	60	34†	54
" ‡	68	57	36†	14
WW	56	34	65	16
"	43	15	81	9
" §	61	22	60	14
"	52	24	60	11
<i>Diabetic</i>				
MS¶	314	298	18	66
NN**	293	261	76	10
CM††	467	453	16	72
<i>Liver disease</i>				
WS‡	72	48	66	3

* 20 g infused except where otherwise stated.

† Exp. interrupted at times given.

‡ 10 g infused.

§ Constant infusion 97 mg/min. after 7.5 g prime. 13 g total amount.

|| " " 136 mg/min. " 4 g " " 12 g " "

¶ 23 yr male requiring 45 u of NPH and crystalline insulin daily.

** 31 " " " 46 u " " " "

†† 54 " " " 36 u of globin & " " " "

tic catheter in the opposite arm before ribose infusion and at 5 to 10 minute intervals after the end of the infusion for a 90 minute period. Two studies were performed in one individual in whom an injection of a priming dose of ribose was given after which ribose was infused at a constant rate with the aid of a Bowman pump. Three studies were also performed in which 16 to 20 g of D-xylose were infused as a 10% solution into 3 normal males. Blood and urine glucose were determined by a method devised in this laboratory (7) similar to that of Froesch and Renold (10) employing the enzyme glucose oxidase. This method measures glucose specifically and does not include non-glucose reducing substances. Ribose was determined by the orcinol method(11) using blood filtrates in which glucose has been destroyed by glucose oxidase. Serum inorganic phosphate was measured by the method of Fiske and SubbaRow(12) and blood pyruvate by the lactic dehydrogenase method of Segal, Blair and Wyngaarden(13).

Results. Effect of infusions of D-ribose

on blood glucose levels. Blood glucose levels fell in all of the normal individuals given D-ribose intravenously (Table I). The magnitude of this fall ranged from 16 to 65% of the initial level. The changes in glucose levels varied from 11 to 41 mg %. The effect of ribose could be reproduced in a given individual as shown on subjects W.W. and J.D.

The pattern of the glucose fall in normal subjects may be seen in Table II, subjects J.D. and W.W. Though slight changes in blood glucose level may be observed at the end of the infusion period (J.D.) usually 20 to 50 minutes before a phase of rapid decline of glucose levels was observed. Minimum glucose levels occurred at 56 to 89 minutes after the start of the ribose infusion (Table I) and subsequently returned to approximately fasting of values at 115 minutes. In the constant infusion experiment shown in Table II (subj. W.W.) the glucose level was depressed after 30 minutes and continued to decrease until 60 to 65 minutes when it was only 48% of the fasting value. This fall occurred during the infusion of only 136 mg of

TABLE II. Levels of Blood Glucose after Ribose and Xylose Infusion.*

Subjects	Duration of infu- sion, min.	Glucose (mg %) at various times (min.) after starting infusion																
		0	15	25	35	45	55	60	65	70	75	80	85	90	95	100	105	115
<i>Ribose</i>																		
JD	25	91		88	89	80	65		65		59	61	73	76	77	79	81	78
WW†	70	52		39		36		24	25	30								
WW‡	11	65	34	9	0	28	26	28	30	33	29	33	45	40	37			
<i>Xylose</i>																		
JD	13	60	73		71	62	67		68									
WR	23	74		95			96		104		98		85					
WM§	115	50	43		50		46				49							48

* 20 g infused except where otherwise stated.

† Constant infusion ribose; 136 mg/min. after 4 g prime; constant blood ribose level 11 mg %; 12 g total amount infused.

‡ 7 U crystalline insulin (0.1 U/kg) given intrav. 4 min. after start of infusion.

§ Constant infusion xylose; 66.5 mg/min. after 10 g prime; constant xylose blood level 40 mg %; 16 g total amount.

ribose/minute.

The blood glucose response in 3 subjects with diabetes mellitus and one with diffuse liver disease is also shown in Table I. Decreases in glucose were seen in the three diabetics. However, only subject N.N. had a pattern of response similar to the normal, the others showing only a transient response at the end of the infusion period. The fall of glucose in subject W.S. with liver disease resembled that seen in the normal subject.

Relationship of blood levels of ribose to hypoglycemic response. The blood ribose concentrations present when the lowest blood glucose levels were recorded are shown in Table I. Low levels of 10-19 mg % are to be noted. Since immediately after infusion of 20 g of ribose the blood levels of ribose ranged from 60 to 80 mg %, the glucose effect cannot be correlated with a high ribose level *per se*. The fact that the time of maximal glucose fall after the single injection of 20 g corresponds with that observed during the constant infusion of small amounts of ribose (Table I subj. W.W.) indicates that the length of time during which ribose is present in the body is an important factor in the glucose responses and suggests that a metabolite rather than ribose itself may be the substance responsible for the hypoglycemic effect.

Changes of phosphate and pyruvate during ribose induced hypoglycemia. Measurement of serum inorganic phosphate and blood py-

ruvate levels was made in four normals whose fall ranged from 39 to 65% and one diabetic (M.S.) with a 5% glucose fall. In the normals the phosphate decreased 10 to 19% and the pyruvate changes varied from a decrease of 34% to an increase of 20%. The diabetic had a fall in phosphate and pyruvate of 19% and 13%, respectively. The decreases in phosphate levels correspond to those observed after infusion of other pentoses(7). The pyruvate changes are probably insignificant. These observations contrast with those seen after insulin induced hypoglycemia in subject W.W. in whom intravenous administration of crystalline insulin (0.1 U/kg) which produces a blood glucose fall similar to that of ribose produced a 39% fall in phosphate and an elevation of pyruvate of 300% of the control value.

Urinary glucose excretion during ribose induced hypoglycemia. Total glucose lost in urine was determined in 2 experiments (subj. J.D. and W.W.). In one, 23 mg of glucose was present in the urine collected during the period when blood glucose fell from 91 to 59 mg %. In the other the loss of glucose in the urine was 75 mg in the period when glucose fell from 43 to 15 mg %.

Effect of D-ribose upon responsiveness to insulin induced hypoglycemia. In one experiment (Table II, W.W.) 7 U (0.1 U/kg) of crystalline insulin was given intravenously through the indwelling catheter while ribose was being infused into the opposite arm and

timed so that maximal hypoglycemia due to insulin would occur about 20 minutes after the end of the ribose infusion. Whereas normally, the blood glucose levels rise to control levels about 90 minutes after insulin administration, in this experiment the blood glucose level was only 57% of the control value 93 minutes after insulin was given, thus indicating that ribose administration had prevented the usual response of blood glucose following insulin hypoglycemia.

Lack of hypoglycemic effect of D-xylose infusion. Since the pentose D-xylose also enters the pentose phosphate pathway to hexose formation(1) three studies with xylose are shown in Table II. Subject J.D.'s response to xylose differs from that to ribose. At the time when low glucose levels are seen after ribose infusion, glucose levels are elevated after xylose. Elevation of glucose levels after xylose infusion is seen also in subject W.R. In subject W.M. during the constant infusion of xylose there was no significant change in blood glucose despite administration of a quantity of xylose equal to that of ribose and the presence of a blood xylose level 4 times as high as the level of ribose at which a hypoglycemic response was noted in the constant ribose infusion study, subject W.W., Table II.

Discussion. The hypoglycemic effects of ribose on blood glucose levels of man may represent an unusual species response. After studying the metabolic effects of ribose administration to both the intact rabbit and mouse, Naito(14) reports that in these species ribose causes an increase in fermentable substance of blood as well as an increase in liver glycogen. It appears also that the effect of ribose on glucose levels in man is specific for this pentose alone and is not a general effect shared by xylose or other pentoses (7).

The mechanism of the fall in blood glucose in man resulting from infusions of D-ribose is at present obscure. It is possible that ribose causes an insulin release from the pancreas. However, the metabolic data of the present study do not support this mechanism as being responsible for the hypoglycemia resulting from infusions of ribose. Injection of insulin into man causes an increase in peripheral

utilization of glucose which is accompanied by large decreases in blood inorganic phosphate (15) and an increase in blood pyruvate(16). Neither of these findings has been observed following ribose administration. Moreover, the fall in inorganic phosphate observed after ribose infusion corresponds to that observed after infusion of other pentoses which have no hypoglycemic effect(7).

In subjects J.D. and W.W. blood glucose levels decreased 32 and 28 mg % respectively. If the glucose space is 17% of the body weight(17) or 12 liters in a 70 kg man, a decrease of this magnitude of blood glucose levels corresponds to a loss of over 3 g of glucose from the glucose space. The urinary glucose loss during these experiments of 23 and 75 mg makes it apparent that the fall of glucose levels due to ribose infusion cannot be due to glucosuria.

The possibility must be considered that ribose alters liver metabolism in such a way as to interrupt the hepatic homeostatic mechanism for maintenance of blood glucose. Recently, potent hypoglycemic agents have been shown to inhibit the breakdown of glycogen in liver(18) and to lower glucose content of hepatic vein blood(19). The observation that ribose may have inhibited the normal compensatory response to insulin induced hypoglycemia suggests that this mechanism may also explain the hypoglycemia resulting from administration of ribose. Should ribose exert its effect by inhibiting hepatic glycogenolysis, a response in the diabetic subject may well depend on the presence of adequate glycogen stores.

Summary. Infusion of D-ribose into normal man causes a marked decrease in blood glucose level which is not associated with significant elevation of blood pyruvate level or large decreases in serum inorganic phosphate concentration. Though the exact mechanism of this hypoglycemia is obscure the data appear to exclude increased peripheral glucose utilization and renal glucosuria as explanations of the ribose effect.[†]

[†] Similar changes to those reported here in blood glucose after ribose infusions into normal individuals have been observed also by Dr. Howard Hiatt, Beth Israel Hospital, Boston, Mass. (personal correspondence).

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Excretion of 17-Ketosteroids in the Rhesus Monkey.* (23287)

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The excretion of 17-ketosteroids in the urine of the female Rhesus monkey was studied in order to determine whether this animal could be used in an experiment investigating adrenal-ovarian relationship and effect of cortisone on menstruation and 17-ketosteroid excretion. Marker isolated 4 mg of Androstenedione(1) from a 6 months pool of monkey urine. Dorfman and associates (2) reported amounts of 17-ketosteroids averaging 1.2 mg/24 hr for the female and 2.1 mg/24 hr for the male in the ketonic fraction of monkey urine, as determined by a Holtorff-Koch(3) modification of the Zimmermann reaction. Furthermore it was observed that Zimmermann chromogen was still present in the urinary extracts following removal of both gonads and adrenals. This led these investigators to the speculation that 17-ketosteroids might originate from a source

other than the adrenals or the gonads.

Methods. Urine from Rhesus monkeys was collected into 2 pools over a period of several days and stored in the refrigerator. The pools measured 1080 ml (3 days) and 1500 ml (approximately 6 days) respectively. The total 17-ketosteroids were determined according to the method of Holtorff-Koch as 12 mg and 20 mg. The following procedures for fractionation and identification were then carried out on both urine pools. The urine was adjusted to pH 1.0 with 50% H₂SO₄ and extracted continuously for 48 hours with ether. The ether was washed with 5% NaOH until pigment-free, then with H₂O until neutral and the washings were added to the urine. Further hydrolysis was achieved by boiling for 30 minutes at pH 1.0. The urine was then extracted manually and washed as above. Both ether fractions were combined and dried over Na₂SO₄. After evaporation

* This work was aided by the U.S.P.H.S.

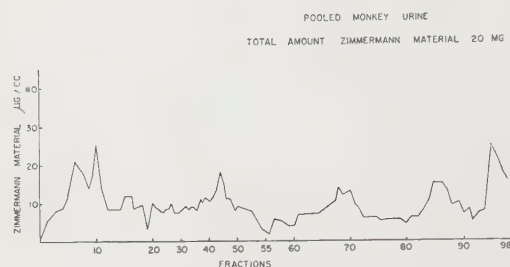


FIG. 1. Distribution of Zimmermann chromogen from monkey urine following chromatography on alumina.

of the ether, residual moisture was removed with benzene. The crude extract thus obtained was chromatographed on alumina in a gradient elution system (Lieberman and Lakshmanan(4)) in which 200 ml benzene and 6% ethanol was added slowly to 800 ml benzene during development of the chromatogram. Removal of more polar steroids from the column was assured by subsequent elution with 20 ml each of 5%, 10%, and 20% ethanol in benzene. The eluate was collected in 100 fractions. Aliquots of 1 ml were taken for Zimmermann reaction (Holtorf and Koch).

Results. Five distinct peaks were obtained (Fig. 1) throughout the chromatograms. Only 2.8 mg of purified Zimmermann reacting material were recovered from the crude extract of the small pool (total 12 mg), while the extract of the larger pool, containing 20 mg of crude material, yielded 5.8 mg. These values were within the range of Dorfman's findings. However, when identification of the material in the peak tubes was attempted by UV, sulfuric acid spectra and infrared spectrophotometry, none of the spectra resembled

those of 17-ketosteroids or other steroids. Moreover, no carbonyl absorption was obtained at $1800\text{--}1580\text{ cm}^{-1}$ and no peaks were observed at $311\text{ m}\mu$ in H_2SO_4 . This led us to believe that the color obtained by the Zimmermann reaction was not specific. It is suggested that the technic used for determination of 17-ketosteroids in humans cannot be applied to monkey urine. This does not imply that the monkey does not excrete 17-ketosteroids, but merely that values given in the literature based on the Zimmermann reaction alone are misleading and have led to erroneous conclusions.

Summary. An attempt was made to study the excretion of 17-ketosteroids in Rhesus monkey urine. No 17-ketosteroids could be detected by ultraviolet adsorption, sulfuric acid and infra-red spectra following chromatography of Zimmermann-material. This suggests that values quoted in the literature, based on Zimmermann reaction alone have led to erroneous conclusions.

POSTSCRIPTUM: Since this paper was submitted a similar discrepancy between Zimmermann reactive material and true 17-ketosteroid content has been reported in pregnant goat's urine by Klyne *et al.* (Klyne, W., Wright, A. A., *Biochem. J.*, 1957, v66, 92.)

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Acetal Phosphatides in Adipose Tissue and Livers of Starved Rats. (23288)

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Previous work reported from this laboratory(1) showed that in the adipose tissue of newborn rats, where this tissue is rapidly filling with fat, the acetal phosphatides and phospholipids, initially elevated, decreased to only trace amounts by the fifth to eighth day of

life. This finding posed the question: would phospholipid and the tissue lipid aldehyde fraction, presumably acetal phosphatides, in the reverse situation, as in starvation, also become elevated during the period of rapid mobilization of depot fat from adipose tissue.

Such evidence might be indicative that acetal phosphatides and phospholipids are involved in some manner in mobilization of fat to and from adipose tissue. For these reasons, changes in acetal phosphatide content of adipose tissue in relation to phospholipid and total lipid content of the same tissue were investigated in rats subjected to starvation and recovery from starvation.

Materials and methods. Twenty-nine female rats, weighing 130 to 160 g of the Carworth Farms strain, were divided into groups of 3, except the first group consisting of 2 animals which served as controls. During an observation period of 5 days, the animals were fed a stock diet of Purina Chow, and their body weights checked daily. The average weight gain/day was about 3 g. After the observation period, all animals, except the control group, were placed on a starvation regime. The animals received nothing to eat, but were given water *ad libitum*. The control animals were sacrificed with no previous period of starvation. At intervals of 1, 2, 3, 4 and 6 days after beginning of starvation period, groups of animals were sacrificed by a blow on the head. While the animal's heart was still beating, the jugular vein was opened and the animal exsanguinated. The liver and perirenal adipose tissue were dissected out, trimmed free of foreign tissue, washed under the tap and blotted dry with paper towel. In dissecting out the perirenal adipose tissue, care was taken to remove the same general area of tissue in each case. At the end of sixth day of starvation period, the remaining groups of rats were presented with food. During the period of recovery from starvation, groups of animals were sacrificed at intervals of 1, 2, 3 and 5 days after first being presented with food. Adipose tissue and livers were removed as described above. As soon after removal of tissue from the body as possible, tissue lipids were extracted by the procedure of Rice and coworkers(2), and the extracts made up to a suitable volume. Aliquots of the extracts were taken for analysis. Acetal phosphatides were determined by the method of Feulgen, Boguth and Andresen(3). This method in our hands, and with palmital dimethyl acetal as a standard for comparison,

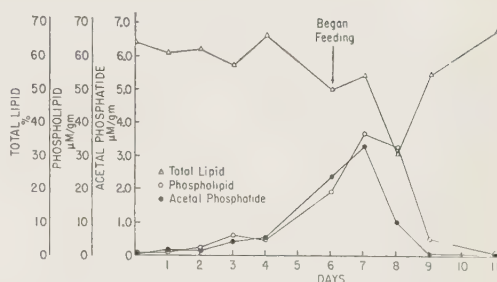


FIG. 1. Lipid changes in adipose tissue of rats subjected to starvation and recovery from starvation.

has given reproducible results with excellent recovery values. Total lipid phosphorus was determined by the procedure of Fiske and Subbarow(4). Total lipids were determined gravimetrically. During the experiment, 3 animals died and some samples of tissue were lost. However, groups of animals were adjusted so that each group consisted of at least 2 animals.

Results. The weight loss of animals during starvation averaged about 10 g/day over the first 4 days, with a total weight loss of 50 to 60 g by the end of the 6 day starvation period. Upon recommencement of feeding, the animals regained the lost weight at the rate of 10 to 15 g/day over the first 3 days, and had regained some 90% of the lost weight by the end of 5 day feeding period.

The changes in liver lipids in starved rats and in rats recovering from starvation are somewhat variable. Total lipid was essentially within normal limits, ranging from 2.86 to 5.07% of the wet weight of tissue. Phospholipids were essentially constant (2.26 to 3.84% wet wt of tissue). Although acetal phosphatides showed a trend toward elevated values during the starvation period, the range of acetal phosphatide concentrations obtained in this experiment (0.112 to 0.725 $\mu\text{mole/g}$ of wet tissue) was within the normal range for liver: $0.365 \pm 0.123 \mu\text{mole/g}$ wet tissue. On the basis of these results, little can be said of the role of acetal phosphatides in liver, although it is possible they may participate in liver lipid metabolism through a turnover mechanism.

In adipose tissue (Fig. 1), the percent total lipid remained essentially constant until the sixth day of the starvation period when

a decrease of 30 to 40% was noted. In this regard, however, the total weight of perirenal adipose tissue available for analysis decreased steadily throughout the starvation period. During the recovery phase, the total lipid content had returned to normal by the end of the fifth day of feeding.

Acetal phosphatides were detectable in only trace amounts (less than 0.05 μ mole/g of tissue) in the adipose tissue of control animals and in animals after one day of starvation. They gradually increased to values of about 0.5 μ mole/g of tissue by the end of the fourth day of starvation, then increased sharply to a peak value of 4 μ moles/g of tissue on the first day of the recovery period. At the end of the second day following recommencement of feeding, there was a pronounced drop in acetal phosphatides which continued to decrease to only trace amounts by the third to fifth day of the recovery period.

Total phospholipids behaved in a manner similar to acetal phosphatides, rising to a peak value on the first day of the recovery period and then falling sharply to only trace amounts at the end of the recovery phase.

The changes in acetal phosphatide and phospholipid in adipose tissue occurring during the first 4 days of the starvation period or the last 3 days of the recovery phase may only represent apparent changes, brought about by changes in other lipid constituents in the adipose tissue, such as neutral fat. However, the level of phospholipid and acetal phosphatides obtained on the last day of the starvation period and on the first day of the recovery phase represents increases ranging from 200 to 1000% for the phospholipid and 400 to 700% for the acetal phosphatides as compared to the levels noted in the control

and experimental animals over the first 4 days of the experimental period. These levels represent real increases since the expected increase due to decrease in total lipid content would be on the order of 100%.

That the mobilization of fat to or from adipose tissue is an active process has been pointed out by Shapiro and coworkers(5). The finding that acetal phosphatides and phospholipids are elevated during the period of most rapid mobilization of fat to or from adipose tissue indicates that they are in some manner involved in this process. Acetal phosphatides could be involved directly in this process, or they could be precursors of other phospholipids which could then be involved in the mobilization of fat. Since mobilization of fat in adipose tissue has been shown to be an active process, acetal phosphatides could possibly also be involved in the production of the necessary energy for transporting the fat across the cell membrane.

Summary. Acetal phosphatides, total phospholipid and total lipids have been followed in the adipose tissue and livers of rats undergoing starvation and recovery from starvation. Whenever fat was being rapidly mobilized to or from adipose tissue, acetal phosphatides and total phospholipids were found to be greatly increased.

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Decreased Gastric Secretory Activity Following Injection of Lignin Sulfonates. (23289)

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We have been studying the effects of certain substances on gastric secretion in the pylorus-ligated rat and Heidenhain pouch dog. While it is premature to report our data for most of these substances, observations on the use of lignin sulfonates, dried and dialyzed pulp mill sulphite waste liquor(1), appear sufficiently definite and interesting to warrant a communication. Since some lignin sulfonate (LS) derivatives prepared here were among the compounds under investigation, we decided to use calcium LS itself in preliminary experiments, the results of which are recorded here.

Materials and methods. Sprague-Dawley rats, starved for 48 hours and deprived of water for the last 16 hours of this period, were lightly anesthetized with ether and the pylorus of each was ligated (The Shay preparation(2)). After ligation, the LS (Ca salt) in water solution was injected into the peritoneal cavity. The controls received an equal injection of water. After 24 hours, the animals were sacrificed and the stomachs removed, emptied and examined. Some of our LS was kindly supplied by Professor Holger Erdtman of the Royal Institute of Technology, Stockholm (S/OMe, 0.38). In most of our experiments, more highly sulfonated material has been used, (S/OMe, slightly less than 0.5) generously provided by Dr. Joseph

L. McCarthy, Department of Chemical Engineering, Univ. of Washington.

Results. In the initial experiment, 6 Shay rats were each given 50 mg of LS (Ca salt) in 0.5 ml of water, as described. Six Shay rats served as controls. When sacrificed, the rumens of the control animals (one had died) were heavily ulcerated. Stomachs from the LS treated animals were without ulcers and had significantly less secretion than the controls. Table I summarizes this and subsequent experiments. An intraperitoneal dose of LS of approximately 10 mg per 100 g rat body weight was a borderline below which there was little 24-hour protection. Three-quarters of this dose at the time of ligation, followed by injection of an equal amount 8 hours later, afforded no protection. Results in the 10-20 mg per 100 g range were generally satisfactory, but for most of this work we used 20 mg per 100 g.

With LS injection 4 to 2 hours before ligation, the anticoagulant action of the LS(3) was so pronounced that many early deaths resulted. Progressively less protection was afforded as time increased between ligation and postoperative injection. With a 4 to 5 hours interval, stomachs 24 hours after ligation were indistinguishable from the controls. This follows from the fact that most of the secretion in the Shay rat occurs during the

TABLE I. Effect of 2 Dosages of LS on Ulcer Incidence and Gastric Secretion in the Shay Rat. Figures in parentheses (last 2 columns) show No. of stomachs represented by preceding avg. Ulcers in test groups were, almost without exception, very small and not bloody, in great contrast to most ulcers in control stomachs.

Rats	No.	Dose CaLS, mg/100 g rat body wt*	Ulcer incidence and description	Avg pH of secretion	Avg vol of secretion, ml†
Controls	11		100% (73% dead or/and perforated)	4.1 (5)	7.8 (3)
Test	14	10	29% (14% dead <i>without</i> ulcers)	4.6 (12)	6.7 (12)
Controls	15		93% (47% dead or/and perforated)	4.3 (8)	8.4 (8)
Test	39	20	18% (8% dead <i>without</i> ulcers)	4.2 (36)	4.7 (36)

* 100 mg LS/ml H₂O.

† After centrifugation.

first few hours following ligation, and ulceration can often be detected within 4 to 5 hours.

In another experiment, 6 Shay rats were each given 0.2 g LS (Ca salt) in 1 ml water *per os* after ligation, and 6 controls were given 1 ml of water *per os*. The following day, although control stomachs were heavily ulcerated (an average of 12 ulcers per stomach was noted, many of them bloody), the stomachs of 4 of the treated animals were completely free of ulceration, one had 2, and one had 5 small ulcers (none bloody). The mechanism of this protection would appear to be different from that after injection of LS into the peritoneal cavity. The average volume of secretion in the stomachs of the treated animals was about the same as in the controls and the pH was more strongly acidic (compare Funk(4)), but in this latter work it should be noted that while ox-bile protected against ulceration, it was also anti-secretory when given *per os* to the Shay rats).

Summary. Solutions of calcium lignin sulfonates injected immediately after ligation in the Shay rat (20 mg/100 g rat weight) afforded pronounced protection against ulceration as compared with controls. The same material given *per os* also protected Shay rats against ulceration, probably by a different mechanism.

We are grateful to Mr. Clifford L. Pitts for technical aid in carrying out these experiments and to the National Institutes of Health for a research grant which aided this work in part.

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Influence of Ascorbic Acid and Chlorpromazine on Body Temperature and Resistance of Mice to Drowning. (23290)

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Increased resistance to anoxia at low barometric pressure resulting from ascorbic acid has been recognized since the report of Gander(1) with Swiss ski troops. Comparable results have been reported with rabbits(2,3), and rats, mice and men(4,5). Because it is known that body temperature influences anoxic resistance to drowning a comparison of body temperature change following injections of ascorbic acid was made. Also injections of chlorpromazine, a hypothermic drug, were used.

Methods and materials. Adult mice of the RAP strain (75 males and 88 females) were divided into groups of 14 or 15. Controls were injected with distilled water of the same volume as that used with the experimentals. Ascorbic acid buffered to pH 7.3 with NaHCO_3 (10 mg in 0.5 ml of water) was in-

jected intraperitoneally and after a lapse of 0, 30, 45, 60, 120 minutes the mice were drowned in water at 37.5°C. Drowning was accomplished by seizing the tip of the tail with a long hemostatic forceps and plunging the mouse under water. The last visible mandibular movement was taken as the criterion of death timed by an electric metronome and observed by at least 2 of the investigators simultaneously. Rectal temperatures were recorded by a L & N potentiometer using copper-constantan electrodes inserted to a marked depth each time. Chlorpromazine (Thorazine S.K.F.*), 25 mg/kg was injected i.p. for the second part of the experiment.

*Chlorpromazine (Thorazine) was kindly supplied by Dr. Edwin J. Fellows, Director of Biological Sciences, Smith, Kline & French Laboratories, Philadelphia, Pa.

TABLE I. Survival of Mice in Seconds of Drowning in Water of 37.5° after Receiving Ascorbic Acid (AA) or Chlorpromazine (CPZ) Intraperitoneally.

No. and sex	Body temp.		Survival (sec.) to drowning after lapse of				
	Before inj.	Before drowning	0 min.	30 min.	45 min.	60 min.	120 min.
15 controls (♀)	37.8 ± .3*	39.0 ± .2	46.6 ± 1.3				
15 AA (♀)	37.1 ± .2	33.6 ± .5		65.3 ± 2.9			
14 "	38.1 ± .2	34.4 ± .5			59.4 ± 2.7		
14 "	36.6 ± .2	35.4 ± .8					57.0 ± 2.4
15 controls (♂)			49.2 ± 1.4				
15 AA (♂)				58.3 ± 1.6			
15 "	†				56.1 ± .9		
15 "						56.3 ± 1.2	
15 "							53.6 ± 1.9
15 CPZ (♀)	37.5 ± .1	32.2 ± .3		71.2 ± 2.9			
15 "	37.6 ± .2	27.8 ± .4					103.0 ± 3.2

$$* \text{S.E.} = \sqrt{\frac{\text{S.D.}^2}{n(n-1)}}$$

† No body temperatures were measured with male mice, as this part of the exp. was done previously and is added here for completeness.

Results. Ascorbic acid lowered body temperature by about 4 degrees in 30 minutes which was maintained throughout the experiment (Table I). There was a gradual increase in temperature with the passage of time until normal temperature had been reached again. At 30 minutes after injection the female mice withstood drowning 40% longer than did the controls, while males survived 18% longer. Both males and females tended to approach survival time of the controls as the elapsed time grew longer. The changes following injection of chlorpromazine were more pronounced than with ascorbic acid. Two hours after injection the body temperature of the mice was only slightly higher than room temperature. The longest survival time occurred 120 minutes after chlorpromazine was injected, 103 seconds as compared to 37.8 seconds for the controls, an increase of 121%.

Discussion. Both ascorbic acid and chlorpromazine produce hypothermia which by itself prolongs anaerobic survival, as shown by morphine, ethyl alcohol and other chemical agents(6,7). Another possibility in prolonging anaerobic survival might be the increase of blood sugar which follows introduction of chlorpromazine(8). It further reduces metabolic rate concurrently with hypothermia which undoubtedly would prolong

survival to anoxia(9). There still is the possibility that some other reaction than reduced temperature and metabolism may be involved.

Summary. Buffered ascorbic acid and chlorpromazine were injected intraperitoneally into mice and the effect on body temperature and anoxic resistance to drowning measured. Significant increases in survival time were noted with ascorbic acid and especially with chlorpromazine. Lowered body temperatures were noted with both compounds. When anoxic survival to drowning was measured, prolongation of life was increased to a maximum of 40% with ascorbic acid and 121% with chlorpromazine.

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Ion Requirements for Gastric Acid Secretion.* (23291)

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Absolute ion requirements for gastric acid secretion have not been determined, although Gray and Adkison(1) and others(2) found that changes in concentrations of Ca, Mg or K in their salt solutions affected secretion by frog gastric mucosae or mouse stomachs *in vitro*. *In vivo* Cl can be almost completely replaced by Br with no adverse effect(3), and mouse stomachs secrete well if 97% of Cl is replaced by Br or I. The law of electrical neutrality of solutions requires that anions accompany the secreted cation, and the mucosa actively secretes Cl in the direction serosa to mucosa(4). There may exist an obligatory coupling between acid and Cl (or halide) secretion. Although Hogben(4) did not find active Na transport in frog mucosa, Rehm (unpublished results) claimed that the dog's stomach pumps Na from mucosal to serosal sides. In the kidney acid secretion appears to be linked with Na transport in the opposite direction(5), and a similar association between acid and Na may occur in the stomach.

To determine ion requirements, O₂ uptake and acid secretion by frog gastric mucosae were measured in a variety of salt solutions in which the ions customarily present in "physiological salt solution" were varied in concentration or eliminated. Frog mucosa was studied because it appears to be less fastidious than mammalian tissue, and it should reveal the minimum requirements of the secretory mechanism.

Methods. Observations were made from January to April on *R. pipiens* obtained from a dealer and kept in a refrigerator. The stomach was removed from a pithed frog, and the muscularis was stripped off and discarded. The mucosa was washed with salt solution and tied and trimmed at junction of stomach and esophagus. The sac was filled with solution and tied slightly proximal to the pyloric sphincter. The filled mucosa was placed in a

large Warburg flask, gassed with O₂ at about 730 mm Hg, and incubated with shaking at 25.2°C. After approximately 15 minutes equilibration measurement of O₂ uptake was begun and continued for an hour when contents of the sac were removed and titrated. O₂ uptake was calculated as qO₂, micromoles of O₂ consumed per mg dry weight per hour, and acid secretion was calculated as qH⁺, micromoles of acid secreted per mg dry weight per hour. These are given in text and tables without repetition of units and with the standard errors of the means. Salt solution used to fill the preparations was an unbuffered one containing 240 mOM of salt calculated without regard to activity coefficients and an additional 11 mM glucose. When changes in composition were made isotonicity was maintained by proper adjustment of the major component. The 10 ml on the serosal side was identical except that it was buffered with 10 mM of tris(hydroxymethyl) aminomethane (Tris). Buffering was necessary, for secretion of alkali on the serosal side otherwise raised the pH to 9 or 10 and reduced secretion. Tris was titrated to pH 7.35 with HCl or other acids, and its osmolar concentration was calculated from its freezing point depression. Concentrations given in the tables are those of solutions as they were prepared. When those of Na and K were measured (by Dr. Richard Malvin) in the serosal solutions at end of incubation the true concentrations are given in parentheses.

Results. *Calcium and magnesium.* Results at top of Table I show that only the Ca and Mg brought to the solution with the tissue are necessary. Addition of 1 mM ethylenediaminetetraacetate (EDTA) abolishes secretion. The effect is reversed by washing the mucosae in 1 mM Ca. Furthermore, inhibition does not occur if EDTA is chelated with a slight excess of Ca. Traces of Ca removable by EDTA are essential for secretion. No conclusion is possible about the role of

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TABLE I. Respiration and Acid Secretion by Frog Gastric Mucosae.

Major salt	Variables, mM	n	qO ₂	qH ⁺
NaCl	1 Ca, .9 Mg, 4.6 K	8	.11 ± .01	.18 ± .02
	0 .9 4.6	6	.12 ± "	.18 ± .03
	1 0 4.6	6	.12 ± "	.20 ± .02
	0 0 3	6	.10 ± "	.17 ± "
	0 0 3 1 EDTA	6	.08 ± "	0
	1.2 .9 3 1 EDTA	6	.13 ± "	.29 ± .03
NaCl	1 Ca, .9 Mg, 0 K (.3)	10	.08 ± .01	.04 ± .02
	1 .9 1 (1.2)	5	.13 ± .02	.22 ± .04
	1 .9 2	6	.11 ± .01	.19 ± .02
	1 .9 3	6	.14 ± .03	.25 ± .03
Choline	0 Na, washed (.6)	6	.06 ± .01	.07 ± .02
	0 (1.3)	12	.07 ± "	.06 ± "
	2 (3)	10	.07 ± "	.06 ± .01
	10	12	.09 ± "	.15 ± .02
	32	11	.10 ± "	.14 ± .01
	111 Li	6	.10 ± "	.14 ± .03
Na	.2 Cl, 116 Br, 2 NO ₃	12	.10 ± .01	.18 ± .01
	.2 116 I, 2	8	.08 ± "	.07 ± .02
	0 118	5	.09 ± "	.11 ± .01
	10 118	5	.08 ± "	.05 ± .02
	32 86	6	.11 ± "	.11 ± "
Na ₂ SO ₄	0 Cl	5	.09 ± .01	.05 ± .01

other metals which chelate with EDTA, for the concentration, if any, of EDTA within the cells is not known.

Potassium. When K was omitted rates of secretion ranged from 0 in four instances up to maximum .12. The serosal fluid at the end of incubation contained .3 mM K. Optimal concentration of K is near 3 mM.

Sodium. NaCl was replaced by choline chloride, and all solutions contained 1 mM Ca, 9 mM Mg and 4.6 mM K. The lowest Na (.6 mM) was attained by washing the mucosae twice. At this concentration secretion ranged from .04 to .14, the highest rate occurring in a solution found to contain .5 mM Na. Raising the concentration to 10 or 32 mM does not restore secretion to maximum. When Li replaced Na as the major cation secretion occurred at two-thirds maximum.

Sodium and adrenal cortical extract. Villarreal, Ganong and Gray(6) found that increased adrenal cortical activity after a latent period of three to four hours was followed by increased acid secretion *in vivo*. We(7) failed to find stimulation of acid secretion *in vitro* by either desoxycorticosterone or cortisone, and desoxycorticosterone at high concentrations inhibited secretion. Some effect

might be found at low Na concentrations where the hormones could promote efficient utilization of the ion. To detect action of any cortical hormones, Upjohn's aqueous extract (generously supplied by Upjohn Co.) was added to both filling and bathing solutions at 1 ml of extract to 100 ml of fluid. The extract used had activity equivalent to .1 mg hydrocortisone per ml, and this gave .011 or more mg of hydrocortisone equivalent per mucosa. Because the extract itself contained NaCl, absence of Na could not be attained. Na concentrations of 5, 12, 32 and 111 mM were used, and the solutions also contained 1 mM Ca, .9 mM Mg and 3 mM K. Results in Table II show that at high Na concentrations the extract reduced secretion, and at low concentrations it had no effect.

Bromide, Iodide, Nitrate and Sulfate. Solutions were made with NaBr, NaI or NaNO₃ replacing NaCl. The source of Ca in all instances was Ca(NO₃)₂. Tris for the serosal solution was titrated with acetic acid to pH 7.35. KBr, KI or KNO₃ was present at 4.6 mM, and results are to be compared with those in the first line of Table I. Histamine phosphate was used in solutions containing 118 mM NO₃ to eliminate halide. Results show that secretion occurs at control level

TABLE II. Effect of Aqueous Adrenal Cortical Extract on Respiration and Acid Secretion of Frog Gastric Mucosae.

Na, mM	n	Controls		Adrenal cortical extract		
		qO ₂	qH ⁺	n	qO ₂	qH ⁺
5	10	.07 ± .01	.11 ± .02	9	.08 ± .01	.10 ± .02
12	9	.10 ± "	.14 ± "	11	.11 ± "	.16 ± .03
32	10	.12 ± "	.18 ± "	11	.10 ± "	.12 ± .02
111	6	.14 ± .03	.25 ± .03	11	.11 ± "	.19 ± "

with Br replacing Cl, but it is reduced with I. Secretion at a reduced rate occurs with NO₃ as the only anion, and replacing NO₃ with Cl to the extent of 32 mM does not increase it. To determine whether secretion can occur with divalent SO₄ as the only anion, the solution on the mucosa side contained 78.3 mM Na₂SO₄, 1.5 mM K₂SO₄, 11 mM glucose and .1 mM histamine phosphate. The serosal solution was the same except that 10 mM Tris titrated to pH 7.35 with H₂SO₄ replaced an equivalent osmotic amount of Na₂SO₄. Ca was omitted on account of its insolubility. In these solutions O₂ uptake was at control levels, and acid secretion definitely occurred although its rate was one-fifth maximal.

Discussion. The results show that secretion can occur when Na or Cl (or halide) are very low in solutions bathing the mucosa. Reduction of secretion below maximal rate under this condition could be explained by lack of essential Na or Cl, and the fact that secretion actually does occur could be attributed to highly efficient utilization of traces of Na or Cl. However, raising the concentrations of Na or Cl does not cause a really substantial increase in secretion until the concentration is more than 10 mM. If Na or Cl is essential one would expect that making more Na or Cl available by raising its concentration only a little would greatly increase secretion. Dependence of acid secretion upon adventitious Na and Cl appears to be relative rather than absolute, and the es-

sential process in gastric acid secretion is the secretion of acid.

Summary. Acid secretion and O₂ uptake measured in frog gastric mucosae *in vitro* occur but at reduced rates when Na in bathing solutions is .6 mM or less. Both are raised towards maximal when Na is 10 mM. Secretion occurs at 75% maximal when Li replaces Na. Adrenal cortical extract reduces secretion with high Na and has no effect with low Na. Neither added Ca nor Mg is necessary for secretion, but secretion is reversibly inhibited by 1 mM ethylenediaminetetraacetate. Secretion occurs when K is .3 mM but is maximum at 3 mM. Secretion occurs at 20% maximal when Cl is .2 mM or less. Secretion is unaffected by replacing Cl by Br, but it is reduced when Cl is replaced by I.

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Inhibition of Lipemia Clearing Activity by Serum of Patients with Hyperlipemia. (23292)

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A defective lipemia clearing system in patients with idiopathic hyperlipemia was demonstrated in the studies of Lever and Waddell(1) who showed a delayed elimination of lipids from the blood stream after an intravenous fat infusion. Inhibition of lipemia clearing by the sera of patients with idiopathic and secondary hyperlipemia is reported. So far, a relation between an inhibitor of the lipemia clearing system and disturbances of lipid transport in humans has not been reported. Seifter and Baeder(2), however, have shown that a dialyzable inhibitor of the lipemic clearing system is present in the blood of rats, particularly following experimental production of a nephrotic syndrome, or following administration of cortisone. Hollett and Meng(3) have separated a nondialyzable component of normal human plasma, which inhibited purified clearing factor completely, while clearing activity in post-heparin plasma was only partially inhibited.

Materials and methods. The degree of inhibition of lipemia clearing activity was tested on sera obtained from 10 patients with idiopathic hyperlipemia, 2 patients with hyperlipemia secondary to glycogen storage disease, 1 patient with hyperlipemia secondary to nephrosis, 2 patients with primary hypercholesteremic xanthomatosis and 10 control subjects. These sera were mixed with normal human or canine serum exhibiting lipemic clearing activity (active serum). Clearing activity had been induced by administering heparin intravenously (0.75 mg of heparin/kg body weight to dogs). No difference was observed whether human or dog serum was used. Equal amounts (0.6 cc) of serum to be tested and of active serum were mixed and 0.3 cc of a standard fat emulsion* was added.

* The standard fat emulsion contained 15% coconut oil, 0.5% Pluronic (a nonionic detergent) and 1% polyglycerol oleate. It was supplied by Upjohn Co., Kalamazoo, Mich.

As an indication of clearing activity, the optical density was measured spectrophotometrically at intervals, as previously described by Grossman(4). In addition varying amounts of hyperlipemic sera were used in a total volume of 1.2 cc to establish at which level complete inhibition of clearing was obtained. Paper electrophoretic analysis was carried out, according to a previously outlined method(5), on mixtures containing hyperlipemic serum, active serum and standard fat emulsion. For comparison of the degree of clearing activity produced by heparin in normal and hyperlipemic subjects 100 mg of heparin were administered intravenously to 4 normal human subjects and to 4 patients with idiopathic hyperlipemia. To 1.2 cc of the respective post-heparin sera, 0.3 cc of standard fat emulsion was added *in vitro* and the optical density of the mixture was measured at intervals as described previously(4).

Results. In contrast to normal sera and those obtained from patients with primary hypercholesteremic xanthomatosis, the sera of all 13 patients with idiopathic and secondary hyperlipemia inhibited to varying degrees the clearing activity which is present in normal sera after the intravenous administration of heparin. When equal amounts of serum to be tested and active serum were used, the decrease in optical density produced in a standard fat emulsion within 120 minutes, varied for the normal and hypercholesteremic sera between 0.23 and 0.33, averaging 0.27 unit; and for the hyperlipemic sera between 0 and 0.17, averaging 0.07 unit (Fig. 1). At sufficiently high concentrations of hyperlipemic serum complete inhibition of clearing activity was observed in all 10 cases. The amounts of hyperlipemic serum necessary to produce complete inhibition of clearing activity varied from 1 part of hyperlipemic to 19 parts of active serum in the most strongly inhibiting serum, to 5 parts of hyperlipemic to

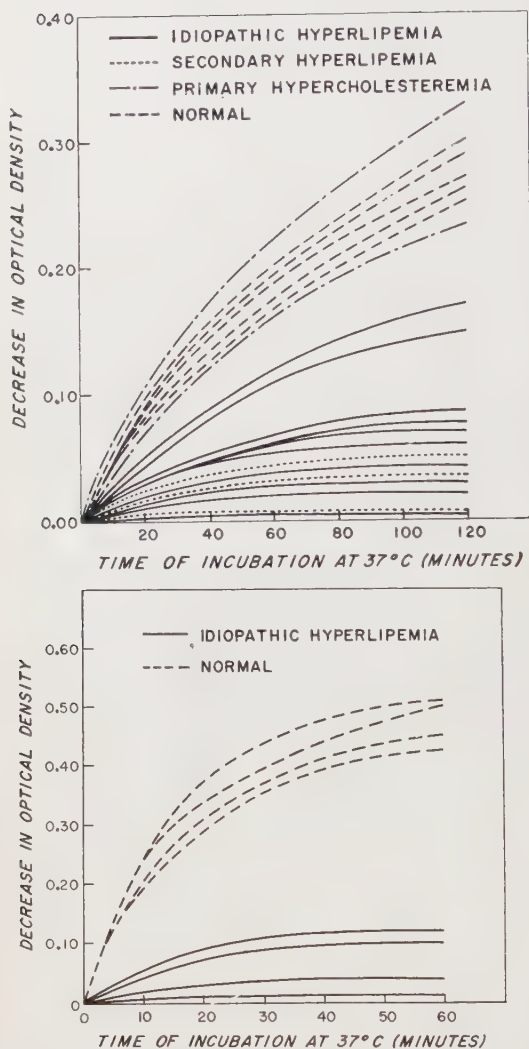


FIG. 1 (top). *In vitro* clearing activity of mixtures of equal amounts of normal post-heparin serum with either normal fasting serum or serum from patients with primary or secondary hyperlipemia or primary hypercholesteremia. (Only 5 of the 10 curves for normal sera are shown. The other 5 curves fell within the same range.)

FIG. 2 (bottom). *In vitro* clearing activity of serum from 4 normal human subjects and from 4 patients with idiopathic hyperlipemia following an intrav. inj. of 100 mg of heparin.

1 part of active serum in the least strongly inhibiting serum. A consistent relationship between the degree of inhibition and severity of the hyperlipemia was not noted. The sera of the 3 patients with secondary hyperlipemia inhibited clearing at ratios of 1 part of hyperlipemic to 9 parts of active serum.

Paper electrophoretic analysis revealed that even in those mixtures of hyperlipemic serum and active serum, in which spectrophotometric measurement showed complete inhibition of clearing, the electrophoretic pattern still revealed accelerated migration of the alpha and beta lipoproteins, typical of the presence of clearing activity.

Determination of the *in vitro* clearing activity, evoked by an intravenous injection of 100 mg of heparin in normal subjects and patients with idiopathic hyperlipemia, revealed much less decrease in optical density and thus less clearing activity in the hyperlipemic patients than in the control subjects (Fig. 2). The decrease in optical density after 1 hour of incubation varied in the 4 normal control persons between 0.38 and 0.49, averaging 0.45 unit; and in the hyperlipemic sera between 0 and 0.11, averaging 0.06 unit.

Discussion. The inhibition of clearing exerted by the serum of patients with idiopathic and secondary hyperlipemia provides an explanation for the high serum lipid levels in these patients. It would be expected that a correlation exists between degree of inhibition and severity of the hyperlipemia. We have not found such a relationship, however. Since dietary intake of fat has a pronounced influence on the lipid levels in these patients, it is likely that in our group of patients who were on varying diets, some on a strict low-fat diet and others not, variations existed in the lipid levels independent of the severity of the inhibition. In order to establish whether a relation exists between degree of inhibition and severity of the hyperlipemia, studies, under standardized conditions, are being carried out.

The observation that the speed of electrophoretic migration of the lipoproteins was increased even when there was complete suppression of clearing and of a fat emulsion, suggests that electrophoretic measurements represent a much more sensitive indicator of clearing activity than optical density measurements.

The greatly reduced post-heparin clearing activity in the serum of patients with hyperlipemia, as compared to normal serum, is further indication of a defect in the clearing mechanism. It is consistent with the obser-

vation of Gitlin (personal communication) that intravenous administration of heparin caused much less clearing activity in the serum of patients with secondary hyperlipemia due to nephrosis, than in normal individuals. He had found that increasing the albumin concentration in these patients who had low serum albumin levels, had no direct effect on degree of clearing. In view of our observation of inadequate clearing in patients with idiopathic hyperlipemia who have normal serum albumin levels, it seems that low values for serum albumin are not a major factor in producing inhibition of lipemia clearing.

Our finding of inhibition of clearing by serum of patients with primary as well as secondary hyperlipemia suggests the possibility of a common basis for the high blood lipid levels in these disorders.

Summary. 1. Serum from patients with primary and secondary hyperlipemia was

found to inhibit the clearing activity present in normal serum after intravenous administration of heparin. 2. The degree of inhibition exerted by the hyperlipemic sera varied among the patients, but at appropriate concentrations of hyperlipemic serum complete inhibition could be attained in every case. 3. Intravenous administration of heparin caused considerably less clearing activity in hyperlipemic patients than in the control subjects.

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Anti-AcG: Specific Circulating Inhibitor of the Labile Clotting Factor.* (23293)

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With recent knowledge that a number of protein factors normally participate in the complex mechanisms of blood clotting, it is understandable that inhibitors might be developed, in the body, to act as specific antibodies against one or other of these protein factors. For instance, anti-AHF or anti-PTC have been demonstrated in occasional hemophiliacs or Christmas disease patients, respectively, particularly after transfusions (1). The present data are from the case of an elderly white male who developed hematuria following an uneventful gall-bladder operation. This bleeding tendency seemed to get worse after transfusions. The following tests establish the presence in his blood of a

powerful inhibitor, specific for AcG (syn. (pro)accelerin, labile factor, factor V, etc.).

Methods are established routines(2), with the modifications indicated. A summary of the routine test findings is shown in Table I (see discussion). The significance of the several abnormal test results will emerge as the experimental analysis proceeds.

Prothrombin time tests. A major initial discovery was the prolongation of the plasma prothrombin time test (*cf.* Quick, 3). This was true also in mixtures of equal vols, of patient's (Y) and normal (N) plasma, immediately suggesting an inhibitor, since normal plasma might be expected to correct the test if it were merely a matter of factor deficiency. Partial, but not complete, correction of the prothrombin time could be obtained by (a) dilution of Y, (b) addition of AcG (BaCO_3 -

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TABLE I. Tests of Patient's Hemostatic and Clotting Mechanisms.

Tourniquet test	Neg.
Bleeding time test	Neg. (4½; 2 min.)
Platelet count/mm ³	Norm. (358-382 × 10 ³)
Whole blood clot.—time	65 min. (prolonged)
Clot retraction	Norm. (no lysis)
Fibrinogen	Norm.
Thrombin clot.—time	Norm.
Protamine titration	Neg.
Prothrombin time	55 sec. (prolonged)
Prothrombin assay	Norm. (122; 126%)
Proaccelerin assay	<1%: anti-AcG + (abnormal)
Proconvertin assay	80%: no anti-proc.
AHF	70%: no anti-AHF
PTC	Norm.: no anti-PTC
P.U.R.	Defic. prothr. consumption
P.T.T.	Abnorm. partial tpln. time
T.G.	Abnorm. tpln. generation

adsorbed beef serum), or (c) mixture of Y's plasma with that of either of his two daughters (in tests made on the last day of hospitalization, when the patient was improving clinically). The daughters' test times were perfectly normal. A beef serum proconvertin (*syn.* SPCA, stable factor, factor VII) did not correct the defect. The above data are illustrated in Table II, for tests made on two occasions (A,B).

Thromboplastin. The above test results were essentially similar with a variety of human or animal thromboplastins. On pre-incubation of plasma (Y) and thromboplastin, before adding the calcium, the clotting-times were further prolonged. This was not significantly changed by adding more thromboplastin, but markedly shorter clotting-times were obtained when AcG was supplied in the calcium solution. *Thromboplastin dilutions.* Modifying the "prothrombin time" test, by varying dilutions of thromboplastin, gave apparent differences between the plas-

TABLE II. Prothrombin Time Tests.

A. 3-25-57		
Normal (N) plasma		11.2 sec.
Patient (Y) "		54.2
Y + N plasma		52.4
Y + AcG		27.2
Y + proconvertin		57
B. 4-3-57		
Daughter (D ₁) plasma		11 sec.
" (D ₂) "		11.2
Patient (Y)		60.3
Y + saline		54.6
Y + D ₁		24.6
Y + D ₂		25.3

mas of patient (Y) and a *fresh* normal (N), as shown in Table III, tests of 3-23-57. A similar difference, at first glance, appeared to be present in tests of 5-8-57, when 1:50 AcG was added to stored (4 weeks at -20°C) Y and to an *aged* (prothrombin time: >60") normal plasma (A). However, increasing the strength of AcG added to Y diminished the difference and in the last series (1:1 AcG) the data were not significantly different from the (A) tests. This argues strongly against any true "antithromboplastin" in Y, but rather points to the very great significance of the AcG concentration in such test series.

Assays for AcG and anti-AcG. AcG is routinely tested by the restoration of clotting in *aged* normal human plasma, which has lost most of its labile factor (test times: >60 sec.). The diluted test sample is added to this substrate, at 37°C, before the thromboplastin (human brain) and the Ca (0.02 M CaCl₂).

AcG. Compared with a fresh normal human plasma, patient Y assayed less than 1%. Further, when samples of (a) bovine serum AcG (above) or (b) fresh N plasma were mixed with Y, the AcG assay values were greatly reduced. This inhibitory effect did

TABLE III. Influence of Serial Dilutions of Thromboplastin (tpln.) on "Prothrombin Time" (Sec., at 37°C). *Test mixtures:* 0.1 ml plasma (or mixture with equal vol AcG, at strengths stated) + 0.1 ml tpln. (at dilution stated) + 0.1 ml 0.02 M CaCl₂. *Plasmas:* Y = patient; N = *fresh* normal; A = *aged* normal.

Tpln. dilution			1/1	1/2	1/4	1/8	1/16	1/32
3-23-57:	Y		31.4"	52.8"	159.0"	360.0"	+	+
"	N		8.9"	11.5"	20.3"	27.0"	35.7"	55.2"
5-8-57:	A + AcG	1:50	14.6"	16.0"	19.0"	21.4"	25.6"	33.6"
"	Y + "	"	30.8"	38.6"	50.0"	64.4"	78.6"	102.0"
"	Y + "	1:5	18.7"	22.6"	29.0"	34.4"	43.4"	56.0"
"	Y + "	1:1	13.8"	15.6"	20.0"	25.2"	29.0"	36.0"

TABLE IV. Titer of Anti-AcG: % Inhibition.

	1:20	1:160	1:320
Citr. plasma	64	22	10
Oxal. "	69	25	14
BaSO ₄ "	69	25	14
(NH ₄) ₂ SO ₄ :			
25% sat., sup.	69	21	
25% sat., sed.	0	5	
33% " "	68	3	
50% " "	72	14	14

not require pre-incubation.

Anti-AcG. The inhibitor *titer* was determined from the reduction in AcG activity on adding serial dilutions of Y's plasma, serum, or fractions to a 1:10 dilution of the bovine AcG preparation. This "standard" AcG was reasonably stable, in an ice-bath, and assayed practically equivalent to fresh normal human plasma. Serial dilutions of the standard AcG served as *reference standard* for converting clotting-times into "percentages" of the original assay strength. Table IV shows typical assay (percentage) anti-AcG values in a preliminary series of fractionations of Y's plasma. The 1:20 dilutions illustrate the comparisons very clearly. Thus, (a) the original plasmas (1:20) gave 64% inhibition in a citrated and 69% inhibition in an oxalated sample. The oxalated plasma was used for the subsequent fractionations. (b) BaSO₄ adsorption failed to remove any anti-AcG, the full 69% inhibition being obtained with the supernatant. (c) After 25% saturation with (NH₄)₂SO₄, no inhibitor was found in the sediment, but the full amount (69% inhibition) was recovered in the 1:20 dil. supernatant. (d) At 33% saturation with (NH₄)₂SO₄, 68% inhibition was shown in the sediment, redissolved to original plasma volume. (e) At 50% saturation with (NH₄)₂SO₄, the redissolved sediment gave negligible further increase (72% inhibition). In all these tests, the inhibitory effect became minimal (<15%) at a titer of 1:160 to 1:320. These preliminary studies suggest that the anti-AcG accompanies some *globulin* fraction. Serum electrophoresis showed certain globulin anomalies which are being analysed. Cryoglobulins were not demonstrable. Table V shows titer testing of the patient's serum, compared with his plasma. Actual

test clotting-times are shown. These were very similar at each successive dilution, indicating that *all* the inhibitor in the plasma survived in the serum after clotting. The plasma and AcG preparation (asterisked*) were tested with saline: all others with standard AcG. Normal human serum did not alter the latter value (15.6 sec.), showing both its lack of AcG and of inhibitor.

Stability data. (a) *Temperature.* The anti-AcG proved remarkably stable at -20°, 4°, 22°, and 37°C. At 37°C, there was no change in titer after 48 hrs, and at 22°C (room temp.) it did not alter in ten days. At 60°C, there were only very minor fluctuations in test values over 1 hr. At 70°C, a progressive loss of inhibitor potency occurred, with practically complete inactivation in ½ hr. At boiling temperature, the anti-AcG was destroyed within 5 min. (b) *pH.* The inhibitor was stable for over 2 hrs between pH 5 and 10. It was slightly weakened at pH 4.0 and 11.5. At pH 2.5, about half its potency was lost in 2 hrs. These tests were performed by adding N/10 HCl or NaOH, using the glass electrode, and restoring to original pH (8.25) before testing, at 1:1 and 1:20 dilutions (accurate volumes). (c) *Fat solvents.* 5 min. shaking with an equal volume of either pure *ether* or *benzene* did not alter the inhibitor test values, whether determined immediately or when retested 24 hrs later.

Discussion. The routine tests, summarized in Table I, may be interpreted as indicating that the patient's blood is deficient in AcG because of the presence of a powerful inhibitor, specific for the labile clotting factor. Other possibilities were ruled out, thus: (a) *Prothrombin:* normal, assaying 122% and 126% by specific one-stage and two-stage

TABLE V. Clotting-Times: AcG Assays.

		Plasma	Serum
Norm.	1: 1	15.5*	(15.6)*
Pat.	1: 0.2	42.	44.4
	1: 2	26.	26.2
	1: 8	22.	22.4
	1: 64	17.	17.5
	1:256	16.6	16.8

* Saline (time in sec.).

tests, respectively. (b) *Proconvertin*: normal, at 80%, with no evidence of any anti-(pro) convertin. (c) The *plasma cofactors* for thromboplastin generation appeared normal, according to preliminary tests, using known hemophilic (AHF-deficient) or PTC-deficient plasmas as substrate and following the prothrombin utilization rate (P.U.R.) in recalcified systems, modified, in the present studies, by addition of AcG (bovine). In these systems, the patient's plasma corrected the substrate defect, and, when Y and N plasma mixtures were tested, there was no evidence of any anti-AHF or anti-PTC. However, P.U.R. on Y's plasma alone (without added AcG), on first examination of this case, was markedly deficient, with only 33% (instead of the normal >90%) prothrombin consumed in 1 hr at 37°C. Another test series, on the last day of hospitalization, gave a normal P.U.R. However, there was much more rapid prothrombin utilization when AcG was also added. (d) *Protamine titrations* were performed on recalcified Y's plasma mixed with serial dilutions of protamine sulfate. The saline control clotting-time, at 37°C, was prolonged to 21½ min. Protamine improved this to 13½ min., but was most effective at very minute concentrations (0.1-0.2 microgram/ml). This, therefore, is similar to most normal plasmas, except for the longer clotting-times throughout. Such a result cannot be taken to indicate the presence of any "heparin-like" factor. (e) The *abnormal* test findings were: 1) lack of AcG and presence of anti-AcG, 2) prolonged clotting-times of whole blood or recalcified plasma, 3) prolonged prothrombin time, 4) prolonged partial thromboplastin time (P.T.T.), *ref.*(4), a 1-stage test in which a brain cephalin preparation is substituted for the brain thromboplastin of the Quick test. 5) Abnormal thromboplastin generation (T.G.). The Biggs-Douglas(5) original test was modified by using brain cephalin instead of platelets. Both the Al(OH)₃ plasma and the serum of patient Y gave abnormal test results. When tests 4)

and 5) were repeated on equal vol. mixtures of plasmas Y and such deficient plasmas as hemophilia (AHF-), Christmas disease (PTC-), Stuart, *ref.*(6), and aged (AcG-), the abnormalities persisted. 6) Abnormal prothrombin consumption (P.U.R.), see (c). The one explanatory common factor is lack of AcG in all these test systems. This lack is imposed, even on reagents originally containing AcG, when mixed with patient's plasma (or serum) supplying the powerful inhibitor. No longer can these test systems perform the purposes for which they are intended. This is both an important piece of evidence of the role of AcG in these systems, and of the disturbing effects of anti-AcG. One previous report(7) in the literature claims anti-AcG as the cause of a bleeding disorder. Many of the present data agree with findings on the German case, but there are a few discrepancies. Some of these may very well be quantitative, but others, such as our complete failure to inactivate the inhibitor with fat solvents, require further elucidation.

Summary. The above data demonstrate an exceptionally interesting and unusual case, namely, a specific inhibitor of AcG (labile factor), occurring in a human subject. Its presence satisfactorily explains the anomalies of a number of clotting tests and accounts for the clinical hemorrhagic syndrome.

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Glucose Utilization and Lactate Production by Leucocytes of Patients with Diabetes Mellitus.* (23294)

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White blood cells are a readily available tissue for the study of cellular reactions in human subjects. Their metabolism has been extensively studied in cells from normal subjects and from patients in a variety of clinical situations, particularly patients with leukemia (1,2,3,4). Martin *et al.*(5) reported that glucose utilization and lactate production are lower in leucocytes from patients with diabetes mellitus than in normal human subjects.

In the present studies utilization of glucose and production of lactate by leucocytes and the effect of insulin on these reactions have been studied in patients with diabetes mellitus and in nondiabetic human subjects.

Procedures. The 9 diabetic subjects attended Third Medical Division Diabetic Clinics at Bellevue Hospital. They ranged from 11 to 58 years and all were fasting when blood specimens were taken. All the diabetic subjects required at least 30 units of insulin/day in the form of regular or NPH insulin. In most cases, an interval of at least 24 hours had elapsed from time of injection of insulin until blood sample was taken. The 13 nondiabetic subjects were medical students or laboratory personnel between 20 and 45 years of age with no known metabolic disease and no family history of diabetes. Experiments were done on both fasting and non-fasting subjects. In 3 cases, blood from non-fasting subjects was obtained during a glucose tolerance test. Venous blood specimens of 30 to 50 ml were obtained using silicone-treated syringes and sodium citrate or heparin as an anticoagulant. Six experiments were run in duplicate on heparin-treated and citrate-treated blood. Level of blood sugar was determined in each specimen and total number

of white blood cells counted. To increase sedimentation rate of the erythrocytes, dextran (average molecular weight 194,000) was added to the blood to a concentration of 0.3 g%. Ascorbic acid was added to a concentration of at least 1.5 mg % (6). Glucose was added in varying amounts to the blood specimens from normal subjects to final concentrations of 115 to 260 mg %. The erythrocytes settled out in the cold, usually in about an hour, and the plasma containing the suspended leucocytes was drawn off. As found by others, the leucocyte suspension always contained erythrocytes (6). On the average, there were approximately equal numbers of red and white blood cells present in the plasma suspensions. This degree of contamination is not serious in these experiments since the glycolytic rate of the leucocytes/cell is about 100 times more rapid than that of the erythrocytes (3). *Measurement of glucose utilization and lactate production:* Aliquots of the leucocyte suspension were transferred to small silicone-treated flasks for measurement of glucose utilization and lactate production. The flasks were shaken under oxygen or air (1) in water bath at 37°C for 2 to 2½ hours. Concentration of glucose was measured by the Somogyi-Nelson method (7,8) and lactate by the method of Barker and Summerson (9). The number of white blood cells present was determined by counting at least 2 pipettes in quadruplicate for each leucocyte suspension. The distribution of the various types of leucocytes was determined by differential white cell counts. In experiments on the effect of *in vitro* insulin, insulin was added to the white cell suspensions in a small volume of sterile isotonic saline. Both glucagon-free and regular crystalline zinc insulin (Lilly) were used. In all experiments, comparable volumes of saline were added to other aliquots of the leucocyte suspensions as controls.

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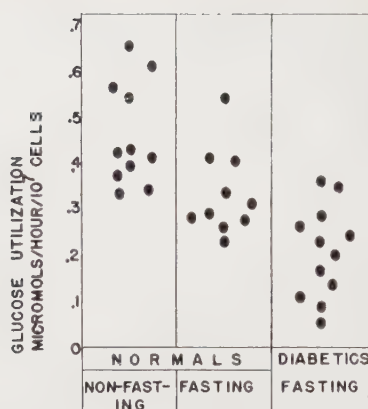


FIG. 1. Rate of glucose uptake by leucocytes of normal subjects and of patients with diabetes mellitus shown in $\mu\text{mols/hr}/10$ million cells at 37°C . Each point represents one experiment.

Results. The rates of glucose utilization and lactate production by leucocytes from normal and diabetic human subjects are summarized in Fig. 1. In the fasting normal subjects, the average rate of glucose utilization was $0.35 \pm 0.03^\dagger$ $\mu\text{mole}/\text{hour}/10$ million cells. In the non-fasting subjects, the rate was 0.45 ± 0.04 $\mu\text{mole}/\text{hour}$. In the diabetic patients who had taken no insulin for 24 hours, the mean rate of glucose utilization was 0.22 ± 0.04 $\mu\text{mole}/\text{hour}$. The difference in rate of glucose utilization between either group of normal subjects and the diabetic subjects is significant by the Fisher "t" test (diabetics compared with fasting normals $p < 0.05$; with non-fasting normals $p < 0.01$). The difference between fasting and non-fasting normal subjects is suggestive but does not meet the statistical criteria of significance. No significant difference in lactate production was observed between any of the groups tested. Lactate production averaged 0.79 ± 0.11 $\mu\text{mole}/\text{hour}/10$ million cells from fasting normal subjects, 1.01 ± 0.13 $\mu\text{mole}/\text{hour}$ by cells from the non-fasting normals, and 0.81 ± 0.12 $\mu\text{mole}/\text{hour}$ by cells from diabetic subjects.

Compared with heparin, sodium citrate was without significant effect on either glucose uptake or lactate production by the leucocytes. In 6 experiments on both normal and diabetic subjects, the average glucose uptake in

heparin was 0.34 $\mu\text{mole}/\text{hour}/10$ million cells compared to 0.35 $\mu\text{mole}/\text{hour}$ in sodium citrate. Values for lactate production were 0.92 $\mu\text{mole}/\text{hour}/10$ million cells in heparin and 0.87 $\mu\text{mole}/\text{hour}$ in citrate.

Distribution of the various types of cells in the leucocyte suspensions was determined by differential white blood cell counts. The proportion of the various types of cells present was within normal limits and did not differ significantly in the 2 groups of subjects.

Effect of insulin *in vitro*: The absolute change in rate of glucose utilization and of lactate production when insulin (up to 0.5 unit/ml) was added *in vitro* is shown for normal and for diabetic subjects in Fig. 2. An estimate of the probable error between duplicate experiments is also included in the Figure. This was computed as one standard deviation above and below the mean difference between duplicate experiments on 2 aliquots of the same leucocyte suspension. (The observed differences between duplicates were assigned positive or negative signs at random.) In about half of the experiments on normal subjects and in more than three-fourths of the experiments on diabetics, the addition of insulin to the leucocyte suspensions was associated with increases in glucose utilization greater than the probable difference between duplicate experiments. The data were also analyzed by the Fisher "t" test, comparing the mean change in rate of glucose utilization where insulin was added to the mean difference be-

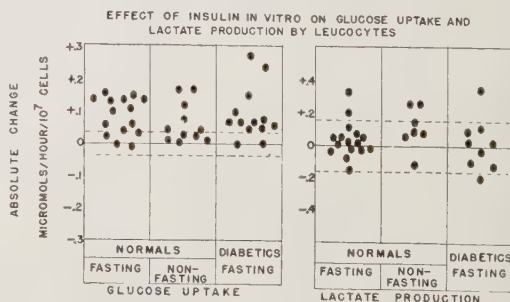


FIG. 2. Effect of adding insulin *in vitro* to leucocytes is shown as the absolute change in rate of glucose uptake and of lactate production in $\mu\text{mols}/\text{hr}/10$ million cells at 37°C . Dotted line indicates stand. dev. of the mean difference between duplicate determinations, when + or - signs were assigned at random to the differences.

† Mean \pm Standard Error.

tween duplicate experiments. In both the normal and the diabetic subjects, the difference in glucose uptake when insulin was added was significant at the $p < 0.01$ level. Insulin had no significant effect on lactate production as shown in Fig. 2.

Discussion. The data presented confirm the work of Martin *et al.*(5) and indicate that rate of glucose utilization by leucocytes is depressed in white blood cells of diabetic patients requiring insulin. In addition, the data suggest that leucocyte metabolism is responsive, in many instances, to *in vitro* insulin. The observation that leucocytes from non-fasting normal subjects tend to have higher rates of glucose utilization than do leucocytes from fasting normal subjects is consistent with considerable evidence that ingestion of carbohydrate normally results in the secretion of additional insulin. Martin *et al.*(5) reported that addition of insulin at concentrations up to 0.1 unit/ml increased glucose utilization and lactate production by leucocytes from diabetic subjects but did not influence these determinations on leucocytes from normal subjects. In our experiments, insulin concentrations up to 0.5 unit/ml were associated with significantly increased glucose utilization in 11 out of 13 determinations on leucocytes of diabetic subjects and in 14 out of 26 determinations on cells of normal subjects. The difference between the results of Martin *et al.* and those reported here is probably related to difference in the concentration of insulin used. Apparently leucocytes from diabetic subjects who require insulin are more likely to be sensitive to *in vitro* insulin than are leucocytes from normal subjects, particularly when the insulin is added at low concentrations.

The surviving tissue in which the effect of *in vitro* insulin has been most thoroughly studied is the rat diaphragm. While there are obvious differences in physical characteristics of rat hemidiaphragms and leucocyte suspensions, it is of interest to compare the rate of glucose utilization and the effect of insulin added *in vitro* to these 2 systems. If the data are expressed in the same units, assuming that 10 million human leucocytes weigh about 6 mg(3), the mean rate of glu-

cose utilization by normal leucocytes incubated in plasma is 10 mg/g (wet weight) of tissue/hour at 37°C. Rate of glucose utilization by the normal rat diaphragm is about half as great, or 4 to 6 mg/hour(10,11,12). When insulin was added to the incubation medium, rate of glucose utilization was increased to about 13 mg/hour by the leucocytes (up to 0.5 unit of insulin/ml), and to 6 to 10 mg/hour by the rat diaphragm (up to 1.6 units/ml), in experiments reported by Gemmil and Hamman(10), Bornstein and Park(11) and Groen *et al.*(12).

There is no necessary contradiction in the observation that glucose utilization by leucocytes is enhanced by insulin and that lactate production is not. Leucocytes are known to contain considerable amounts of glycogen which can serve as a source of lactate(13). Shaw and Stadie(14) have reported experiments on the rat diaphragm which suggest that lactate production from glycogen is not responsive to insulin.

Summary. Glucose utilization and lactate production have been measured in suspensions of leucocytes in plasma from normal human subjects and from patients with diabetes mellitus. The rate of glucose utilization was significantly lower in leucocytes from severe diabetics than in leucocytes from normal human subjects. Lactate production did not differ significantly between normal and diabetic subjects. In more than three-fourths of the experiments on diabetics and about one-half the experiments on normal subjects, glucose utilization by leucocytes was increased by insulin *in vitro*. Lactate production was not increased when insulin was added *in vitro*.

The author is indebted to Dr. Elaine P. Ralli for the opportunity to study patients in the Diabetic Clinics, and for advice and encouragement. The assistance of Mr. Bertram Laken in the lactate determinations is also gratefully acknowledged.

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Vitamin A-Choline Interrelationship.* (23295)

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Abels, Gorham, Pack, and Rhoads(1) found that levels of vit. A in plasma of patients with malignant disease, particularly of the gastrointestinal tract, were below the normal range in 86% of cases examined. No response was obtained after vit. A administration, but feeding of yeast or pancreatic extracts, themselves free of carotenoids, raised the reduced plasma levels of vit. A. That this property in yeast or pancreas may be due to choline content has become apparent from later experiments. There was an average rise of 73% in plasma levels of vit. A in patients who were fed 1.5 g of choline chloride each day for 3 days. Popper and Chinn(2) showed that rats on a choline-poor diet containing liberal supplements of carotene developed fatty livers poor in vit. A. Therefore, it seemed worth while to determine choline levels of rat livers in normal, vit. A-supplemented, and vit. A-deficient animals.

Methods. In general, the procedure was to determine the amount of vit. A and choline in separate aliquots of rat livers from different dietary groups. Male, albino, Wistar-strain rats were used. The rats were divided into 3 dietary groups, normal (N) supplemented (S), and deficient (D). All animals were placed on a Red Range Dog Food† ra-

tion for at least 2 weeks. Animals of the supplemented group received an additional 5,000 International Units of vit. A‡ orally each day for one week before sacrifice. The deficient group was maintained on a vit. A test diet§ up to 7 weeks at which time vit. A analyses indicated that only trace amounts of vit. A remained in the liver. Each group was fasted for 24 hours before sacrifice by decapitation, accompanied by gravity exsanguination for approximately one minute. Usually 3 rats from the same dietary group were sacrificed at one time and their livers pooled. The livers were removed and placed in a freezer, at 10 to 15°C. The livers were then placed in a pre-cooled flask and weighed. The vit. A levels were run on freshly-chilled portions of liver, while total and phospholipid choline determinations were run on frozen livers. The livers, for vit. A analysis, were minced and one-half to one g portions were placed in a Potter-Elvehjem homogenizing tube containing 5 to 7 ml of ice-cold (0-5°C) distilled water. The homogenate was transferred to a 100 ml volumetric flask, and more liver was homogenized until 3 to 6 g of liver had been homogenized. The total homogenate was diluted to 100 ml with more cold distilled water. Triplicate aliquots of the homogenate were used immediately for determination of vit. A,

* This work was supported in part by Atomic Energy Commission contract.

† Red Range Dog Food sold by Southern States Feed Cooperative. Average Analysis (%), Protein 24.60, Fat 5.04, CHO 58.12; Vit. content/100 g, Vit. A activity-1100 I.U., Carotene 0.14 mg, Choline chloride 7.13 mg.

‡ Aqueous solution of vit. A prepared by Endo Products Inc. containing 25,000 I.U. of vit. A/ml of solution.

§ U. S. Pharmacopeia vit. A test diet distributed by Nutritional Biochemicals Corp. Average choline content 20 mg/100 g.

TABLE I. Total Choline, Phospholipid Choline, and Calculated Free Choline in Rat Livers on Normal, Deficient, and Supplemented Vit. A Diets.

Dietary group	Avg rat wt (g)	Liver vit. A ($\mu\text{g/g}$ wet liver)	Total choline	Phospholipid choline	Calculated free choline
			(mg/g wet liver)		
N-1	239	184.2	3.12	1.96	1.16
2	105	170.0	3.31	2.30	1.01
3	116	182.0	3.21	2.03	1.18
D-1	129	32.3	2.57	2.04	.53
2	179	6.3	2.21	1.93	.28
S-1	114	483.5	2.50	1.86	.64
2	119	437.8	2.26	1.53	.73
3	143	480.0	2.71	2.10	.61

by the procedure of Oser *et al.*(3). Determination of total choline, by the procedure of Engel(4), was run in duplicate on samples of frozen livers, which ranged from 1.5 g to 2.5 g wet liver. For determination of phospholipid choline, the procedure of Entenman *et al.*(5) was used. The color produced by choline reineckate was measured on a Coleman Junior Spectrophotometer at a 520 m μ wave length setting. The standard used was prepared according to Beattie(6). Concentration of the standard corresponded to 0.26 mg choline chloride/ml.

Results. The results are presented in Table I. The μg of vit. A/g wet liver are given for the 3 dietary groups. It will be noted that the vit. A levels of the 2 deficient groups (6.3-32.3 μg) show only trace amount of vit. A, while the concentration of vit. A (437.8-483.5 μg) in the supplemented rats is well above that of the normal groups (170-184.2 μg).

Total choline, in mg of choline/g wet liver, was highest in normal groups, ranging from 3.12 to 3.31 mg. The lowest concentrations of total choline were reported in a deficient group (2.21 mg) and a supplemented group (2.26 mg).

Phospholipid choline did not vary as much as did total choline. The highest level was in a normal group with 2.3 mg and the lowest in a supplemented group with 1.53 mg.

The free choline was calculated by subtracting the phospholipid choline from the total choline.

From the data in Table I, it appears that the optimum level of choline in the liver is associated with a normal liver level of vit. A.

Low or abnormally high liver vit. A levels are associated with a low level of choline in rat livers.

Discussion. The results indicate that there is some vit. A-choline interrelationship. The total choline concentration was highest in the 3 normal groups, but this concentration was reduced when an excessive amount or a deficient amount of vit. A was stored in the liver. Since the phospholipid choline content showed little variation under these experimental conditions, it appears that total choline variation is on the basis of a change in the free choline content. It is interesting to note that the maximum level of free choline is related to the normal level of vit. A.

This relationship between vit. A and choline has been further substantiated by the work of Thorbjarnarson and Drummond(7), who found that choline feeding stimulated removal of vit. A as well as fat from the liver. Popper and Chinn(2) also reported that a choline-poor diet revealed a similar dependence upon vit. A storage in the liver.

The low vit. A blood levels found in patients with malignant disease of the gastrointestinal tract may in some way be tied up with a disturbance of this vit. A-choline relationship in the liver.

Summary. The highest level of free choline in rat livers was obtained from rats with a normal level of vit. A storage in their livers. Rat livers which contained an excessive amount or a deficient amount of vit. A had low concentrations of free choline.

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Lymphocytosis Response in Mice and Its Relation to Thymus and Adrenal.* (23296)

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During the course of observations on the effect of thymectomy on circulating lymphocyte levels in the mouse, it was observed that sham-operated mice did not maintain stable white cell levels as expected, but invariably developed a lymphocytosis. Subsequently, it was found that normal mice developed a similar lymphocytosis when bled daily for the performance of white cell counts. This paper describes this lymphocytosis response in mice, its variation in mice of different strains and experiments throwing light on the mechanism by which the response is produced.

Materials and methods. *Mice.* Mice used throughout these experiments were obtained from the Jackson Memorial Laboratories and were of the following strains: AkR, Rf, C₅₇Bl, LAF₁, and C₅₈. All mice were fed on purina chow and maintained on oral terramycin to prevent intercurrent infections. Male mice were used throughout these experiments to eliminate possible white cell variations with the estrus cycle. However, some groups of female mice were tested to make sure no notable difference was present in the lymphocytosis response of the two sexes. No difference was observed. The age of the mice used was 4-5 weeks. All mice were autopsied at the conclusion of each experiment to check on the adequacy of the operative procedures. Results from any mice showing infections were discarded. *White cell counts.* Standard

human white cell diluting pipettes and chambers were used. Differential white cell counts were made, counting 200 cells. Blood was obtained by pricking the tail veins with a sharp No. 13 scalpel blade. "Milking" of the tail to produce a flow of blood was avoided as was making too large a wound with the production of too free a flow of blood. Careful standardization of the bleeding procedure is necessary because of the difference between the absolute numbers of white cells in the peripheral and heart blood in the mouse(1). White cell counts were performed at fixed times during the day to eliminate any possible diurnal variations. Due to individual variations in white cell levels even within inbred strains, groups of 4 to 6 mice were used in each experiment, and the mean value for the group used as a single estimation. *Operations.* When required, thymectomy and/or bilateral adrenalectomy were performed, using I.P. nembutal anesthesia. Adrenalectomized mice were maintained on a single I.M. injection of 0.5 mg of percorten[‡] and 0.5% NaCl in the drinking water.

Results. *The lymphocytosis response.* When daily white cell counts were performed on the tail blood of normal mice of C₅₇Bl strain, a lymphocytosis appeared on the second day. This persisted for as long as daily observations were made. The lymphocytosis levels fell towards the baseline levels of randomly bled mice several days after cessation

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[†] Carden Fellow in Cancer Research Anti-Cancer Council of Victoria.

[‡] This preparation (Percorten trimethylacetate) was generously supplied by Dr. Robert Gaunt, Ciba Pharmaceutical Products, Summit, N. J.

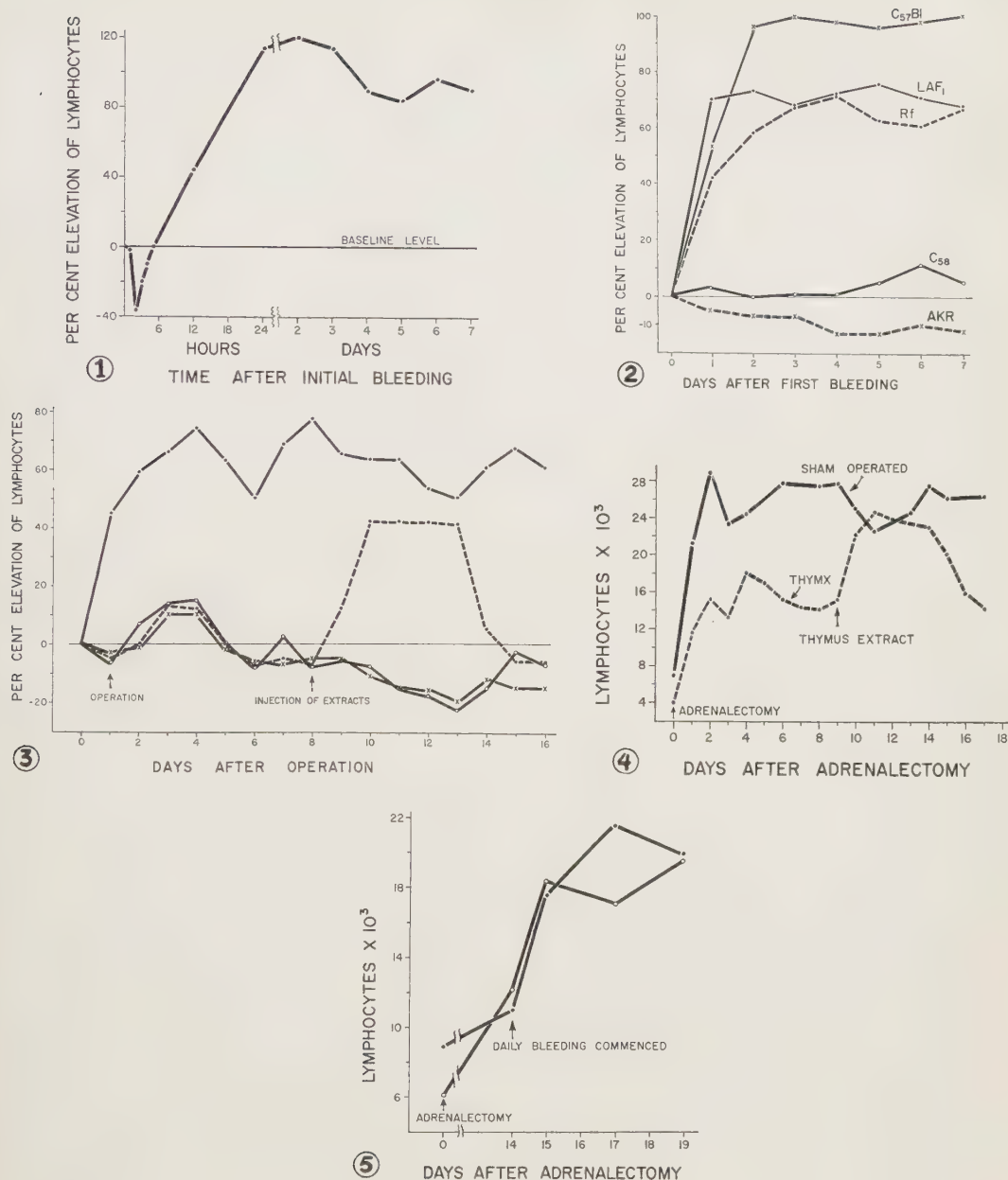


FIG. 1. Lymphocytosis response of repeatedly bled young male mice of Strain C₅₇Bl. Each point represents mean lymphocyte count of 6 mice.

FIG. 2. Effect of daily bleeding on circulating lymphocyte levels in mice of various strains. Each point represents mean lymphocyte level in 20 mice of each strain.

FIG. 3. Effect of thymectomy and sham thymectomy on circulating lymphocyte levels. Each point represents mean lymphocyte level in 20 mice. Mice were young male adult Rf strain. Inj. of organ extracts was made on 8th post-operative day. ●—● sham thymectomy; ○—○ thymectomy; ●---● thymectomy + thymus extract; ×—× thymectomy + lymph node extract.

FIG. 4. Effect of adrenalectomy and thymus extracts on circulating lymphocyte levels in thymectomized and sham-thymectomized mice. Each point represents mean lymphocyte level of 8 mice.

FIG. 5. Effect of adrenalectomy on lymphocytosis response. Mice were young male adult Rf strain. Graph shows mean results from 2 identical groups of 4 mice each.

of daily bleeding. The level of the lymphocytosis reached varied from group to group of mice, but usually represented a 50-120% increase over baseline values. The polymorphonuclear levels remained relatively constant, with minor daily fluctuations, unless intercurrent infections or fluctuations in room temperature occurred.

A more detailed analysis of the changes following the performance of the first white cell count showed that there was an initial fall in lymphocyte levels lasting for approximately 2 hours (Fig. 1). This lymphopenia probably represents a response to adrenal-mediated acute stress. From this point on the lymphocyte levels rose progressively, reaching a maximum by 20-36 hours.

Strain differences in lymphocytosis response. The sequence of events described above was found to hold true for the following strains of mice: C₅₇Bl, Rf, and LAF₁. Of these, the C₅₇Bl strain tended to show a more pronounced rise than did the other strains.

When AkR and C₅₈ strain mice were bled daily, no lymphocytosis occurred, although the initial lymphocyte levels of these mice were consistently higher than for the other strains. With mice of the AkR strain, a definite fall in lymphocyte levels occurred (Fig. 2). It should be noted that the AkR and C₅₈ strains are characterized by a high incidence of spontaneous lymphatic leukemia, in contrast to the other strains studied.

Mechanism of the lymphocytosis response. The stimulus arousing the lymphocytosis response is associated with wounding of the skin of the tail during the bleeding process. When the initial wound was made through the skin away from the site of a tail vein, with no accompanying blood loss, the lymphocytosis response occurred as before. The effect does not, therefore, appear to depend on either the stimulus of blood loss or stimuli from damaged blood vessels.

It was of interest to determine the effect of thymectomy on this response since it has been shown(2) that the thymus produces a lymphocytosis stimulating factor (LSF). Thymectomies were performed on groups of mice as described above, and serial daily white cell counts performed. The mice failed

to show a lymphocytosis but instead showed a slight but definite fall in lymphocyte levels. Sham-operated mice showed a similar lymphocytosis to that occurring in normal animals bled daily. When thymectomized mice were injected with saline extracts of thymus, a temporary lymphocytosis of the same magnitude as that in sham-operated mice resulted. Thymus extracts from strains of mice other than the strain of the thymectomized mice were also used, and the extracts centrifuged at 3000 rpm to minimize introduction of intact lymphocytes. The injection of these thymic extracts was also followed by a lymphocytosis. Injection of similar extracts of lymph node or spleen produced no such effect (Fig. 3). A similar lymphocytosis was elicited by injecting thymus extract immediately following thymectomy. This indicates that the response of thymectomized mice to injections of thymus extract is not dependent on compensatory hyperplasia of remaining lymphoid tissue in the animal. In fact, no such lymph node hyperplasia was observed in thymectomized mice which were killed and examined at the termination of the experiments.

These findings confirm earlier work(2) indicating that the epithelial type cells of the mouse thymus medulla contain the lymphocytosis stimulating factor (LSF), and suggest that in the lymphocytosis response, following tail wounding, thymic release and production of LSF occurs causing a lymphocytosis.

In view of the known lymphopenia-producing effects of adrenal cortex extracts, it was thought that adrenal corticoids might normally suppress thymic LSF activity and that under the conditions producing the lymphocytosis response, a partial or complete release of the thymus from this adrenal suppression might occur. To test these possibilities, bilateral adrenalectomies were performed on groups of thymectomized and sham-operated animals. Normal mice were also adrenalectomized and later tested for their ability to show a lymphocytosis response.

It was found (Fig. 4) that adrenalectomy caused a lymphocytosis both in sham-operated and thymectomized mice. However, the levels reached in thymectomized mice were

consistently lower than in the sham-operated mice. This indicates that the adrenal normally suppresses thymic production of LSF and, consequently, depresses the level of circulating lymphocytes. Adrenalectomies were then performed on normal mice and a period of 2 weeks allowed to elapse to enable wound healing, and stabilization of lymphocyte levels. These mice were then bled daily.

Following the commencement of daily tail bleeding, a lymphocytosis response occurred, superimposed on the pre-existing elevated lymphocyte levels (Fig. 5). These findings suggest that, although the adrenal may normally suppress thymic LSF activity, release from this adrenal suppression plays no part in the development of the lymphocytosis response.

Discussion. The lymphocytosis response appears to be a normal response of the mouse to trauma—in the experiments described above, that involved in tail bleeding or in operative wounding. Elevated lymphocyte levels persist for as long as the trauma is repeated and pass off several days after the last injury. Polymorphonuclear levels are not altered significantly during these changes.

Striking strain differences were found in the ability to develop the lymphocytosis response. The high leukemia AkR and C₅₈ strains appeared unable to develop a lymphocytosis when stimulated, although the initial lymphocyte levels were higher than all other strains examined. The relation of these findings to the pathogenesis of lymphatic leukemia in these mice is not known, but it has been shown(2) that elevated thymic LSF levels are present in mice with spontaneous lymphatic leukemia.

Thymectomy prevents the development of a lymphocytosis response. This is not the non-specific result of removal of a considerable fraction of the mouse's lymphopoietic

tissue. Injections of relatively cell-free saline extracts of thymus tissue from even a foreign strain enable thymectomized mice to develop a lymphocytosis similar to sham-operated mice. This effect does not follow injection of similar preparations of lymph node or spleen, indicating that it is not due to the injection of intact lymphocytes or material from disintegrated lymphocytes.

Adrenal corticoids in the normal mouse appear to suppress the number of circulating lymphocytes both by a direct process and an indirect process via the thymus. In unpublished experiments, cortisone, when injected into mice, has been found to depress the thymic content of LSF. However, removal of this adrenal suppression of thymic LSF production appears to play no part in the lymphocytosis response, as the response occurs in adrenalectomized mice.

The lymphocytosis response, therefore, would seem to be initiated by stimuli arising from damaged tissue and appears to be mediated through thymic LSF. Whether this is a direct stimulation of the thymus or an indirect one, via other organs, is yet to be determined.

Summary. (1) Mice of certain inbred strains (C₅₇Bl, Rf, and LAF₁), when bled daily from tail veins, developed a prompt and persistent lymphocytosis. (2) High leukemia strains AkR and C₅₈ did not show this response. (3) Thymectomy prevented this response but lymphocytosis was produced in thymectomized mice by injection of cell-free thymus extracts. Lymph node and spleen extracts were ineffective. (4) Adrenalectomy did not affect the lymphocytosis response.

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Resistance to Endotoxin After Protection Against Initial Lethal Challenge with Adrenocorticosteroids or Chlorpromazine. (23297)

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Previous investigations have established that mice may be protected against lethal doses of brucella endotoxin by both adrenocorticosteroids(1,2) and chlorpromazine(3). It has also been observed that injection of a single sublethal dose of brucella endotoxin confers resistance to subsequent challenge with lethal amounts of endotoxin(4). Such resistance was found to develop within 2 weeks and to persist for approximately 6 months. The studies reported here were done to determine if therapy with steroids or chlorpromazine interfered with the development of subsequent resistance to the lethality of endotoxin.

Materials and methods. Male ABC mice weighing 20 to 25 g were housed 5 to 10 animals per cage in an air-conditioned room and had free access to tap water and Purina Fox Chow. The endotoxin was prepared from *Brucella melitensis* by a modified Boivin technic(5). A saline suspension was injected intraperitoneally. Appropriate dilutions with saline of the protective agents were given intramuscularly.

Results. Various groups of mice were treated with either cortisone acetate, 9-alpha-fluorohydrocortisone acetate, or chlorpromazine by the schedules outlined in Table I. These groups, along with control groups given injections of sterile saline at the same intervals, were then challenged with 2 LD₅₀

amounts of brucella endotoxin. With each agent, significant protection against endotoxin lethality was observed. Following this initial challenge, the survivors from each treatment group were then subsequently challenged with a lethal amount (2 LD₅₀) of endotoxin at either 2 or 4 weeks. Again, untreated mice of the same approximate weight were challenged as controls. The results (Table I) indicate that the protected animals developed resistance to endotoxin lethality by 2 weeks and that this resistance persisted at least 4 weeks.

Discussion. These findings show that protection against an initial lethal dose of endotoxin with adrenocorticosteroids or chlorpromazine does not interfere with the development of resistance to endotoxin. Such an observation might have significance in the treatment of serious infections due to Gram-negative micro-organisms. It is now well established that the febrile and toxic state that occurs in such infections can be controlled and subdued with adrenocorticosteroids, though the mechanisms responsible for this beneficial effect are not understood(6).

Summary. Mice protected against an initial lethal challenge with brucella endotoxin by treatment with cortisone acetate, 9-alpha-fluorohydrocortisone acetate, or chlorpromazine develop resistance to subsequent challenge to endotoxin.

TABLE I.

Agent	Schedule of treatment	Endotoxin mortality*					
		Initial challenge		2 wk later		4 wk later	
		Treated	Controls	Treated	Controls	Treated	Controls
Cortisone	.5 mg I.M. 3 hr†	8/20	8/10	3/ 9	10/10		
"	<i>Idem</i> 2 hr†	10/45	14/15	1/15	9/12	1/15	8/10
9-alpha-fluoro-hydrocortisone	.05 mg I.M. 2 hr†	15/25	10/10	2/ 9	10/10		
Chlorpromazine	.2 mg I.M. every 4 hr × 4	2/10	7/10			2/ 8	10/10

* No. dying/No. challenged.

† Before challenge.

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Dehydrogenase Enzyme Studies in Experimental Renal Hypertension.* (23298)

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Previous investigations(1,2,3) have demonstrated that the kidney, adrenal, thyroid and pituitary play a role in development and maintenance of experimental renal hypertension. In the present investigation one facet of metabolic activity of these organs was measured in an attempt to determine if an abnormal metabolism within one or more of them could be related to the etiology of experimental renal hypertension, while cardiac muscle was likewise studied to observe any effects of high blood pressure levels on this portion of the cardiovascular system. Since reserpine effectively lowers the hypertensive blood pressure level in experimental renal hypertension(4), it was decided also to study the above mentioned tissues in hypertensive animals under active reserpine therapy. The area of tissue metabolism studied was the endogenous dehydrogenase enzyme activity. This was quantitated employing the tetrazolium technic.

Methods. Both male and female rats of the Denver strain were used. During the years this colony has been employed for cardiovascular problems in our laboratory, the normal, mean arterial blood pressure, measured directly under general anesthesia, has been determined to range from 100-140 mm Hg. With this information available the

lower limit for a hypertensive blood pressure level was set at 150 mm Hg. Experimental renal hypertension was produced in 3 different ways. Constriction of the remaining kidney by means of a figure 8 ligature after unilateral nephrectomy(5), feeding a choline deficient diet early in life(6), and encapsulation of one kidney followed by removal of the contralateral kidney(7) were the methods employed. For the latter procedure a butyl-methacrylate polymer plastic dissolved in acetone was used to construct the capsule. Blood pressures were measured indirectly using the "Infraton" sphygmograph system(8) and recorded on a direct writing Sanborn E.C.G. Readings were obtained with animals under light ether anesthesia. Measurements using this method were found to agree favorably with simultaneously recorded direct blood pressure measurements. Only animals which were acutely hypertensive were employed for the endogenous dehydrogenase enzyme determinations. Following the first complete week in which an animal presented a consistently elevated mean arterial pressure, as measured every 2 days, the animal was sacrificed or placed upon reserpine therapy for 14 days before being sacrificed. A dose of 100 μ g/kg/day of reserpine was necessary to reduce the elevated blood pressure to a stabilized level within 14 days. Pure powdered reserpine was used.[‡] It was dissolved in a minimum of glacial acetic acid and brought to the

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[‡] The powdered reserpine (Serpasil) was obtained through the courtesy of Dr. F. F. Yonkman.

TABLE I. Comparative Endogenous Dehydrogenase Enzyme Activity of Tissues from Normal, Hypertensive, and Reserpine Treated Hypertensive Rats.

Exp. procedure	No. of animals	Avg mean arterial pressure of group	Mean values (μ g of dye reduced/mg air dried tissue)				
			Kidney	Adrenal	Thyroid	Pituitary	Heart
Normal	17		36.7	22.9	11.0	6.4	17.3
" + reserpine	14		32.6*	23.7	8.2†	5.6	14.4*
Renal constriction	14	159	32.1*	25.0	10.7	7.6	17.9
<i>Idem</i>	9	193	32.4*	24.6	10.9	7.8	19.7*
Renal encapsulation	11	198	30.6*	21.2	7.9*	4.0	18.1
<i>Idem</i> + reserpine	11	143	30.0	22.4	8.1	6.0	14.2†
Choline deficiency	7	185	34.7	22.6	9.1	7.0	20.8*
<i>Idem</i> + reserpine	6	137	31.5	23.7	9.2	6.6	13.8†

* $P < .01$ when compared with means of normal animals.

† $P < .0001$ when compared with means of animals made hypertensive by the same procedure but receiving no reserpine.

‡ $P < .02$ but $> .01$ when compared with mean of normal animals.

desired volume with a 10% propylene glycol medium and administered subcutaneously. This same dosage and time schedule in normal rats resulted in some anorexia and weight loss. The endogenous dehydrogenase enzyme activity was quantitated using the method of Kun and Abood(9) as modified for tissue slices(10). Triphenyltetrazolium chloride was used as the indicator in a modified Kreb's solution(11). Incubation was carried out at 37° for 2 hours. The entire pituitary and both lobes of the thyroid were incubated without further sectioning, while the adrenals were carefully divided with a razor blade. Tissue slices approximately 1-2 mm x 1 cm x 1 cm were cut freehand from the kidney and heart. The amount of dye reduced by each piece of tissue was determined colorimetrically using a Bausch and Lomb Spectronic "20", and was then converted to μ g of dye reduced/mg of air dried tissue for comparative purposes. Animals made hypertensive by renal constriction with a figure 8 ligature were divided into 2 arbitrary groupings on the basis of level of blood pressure reached. Those animals in the first group had a blood pressure between 150-180 mm Hg, while in the second group the blood pressure was greater than 180 mm Hg for each animal. This was done to observe if any difference in the dehydrogenase enzyme activity of the tissues could be related to the level of blood pressure acutely attained.

Results. The results obtained are tabulated in Table I. In the statistical analysis,

the mean values obtained for each tissue in the 2 hypertensive groups treated with reserpine were compared only to the groups in which hypertension was similarly produced but left untreated.

Of the organs studied, the adrenal and pituitary showed no significant change for any of the experimental groups. Although the pituitary appears to have a low value for the group in which hypertension was produced by renal encapsulation, it was not statistically significant.

In the case of the thyroid the results were equivocal. The endogenous dehydrogenase enzyme activity was lower than normal in those groups made hypertensive through feeding of a choline deficient diet early in life and by renal encapsulation. The latter lowering was statistically significant. Since this same condition did not result in the group in which hypertension was produced by renal constriction, it can not be said that any significance can be attached to this lowered activity in relation to the etiology of experimental renal hypertension. Administration of reserpine also lowered the endogenous dehydrogenase enzyme activity of the thyroid. This condition is being further investigated by us to determine the mechanism of action for this reduction.

All of the experimental procedures producing hypertension resulted in a reduction in endogenous dehydrogenase enzyme activity of the kidney. A reduction in succinic dehydrogenase enzyme level of kidney tissue from

animals made hypertensive by partial renal artery occlusion has previously been reported (12). The present investigation further suggests that the kidney is involved in the etiology of experimental renal hypertension at a metabolic level. The exact mechanism whereby the kidney produces its action cannot be determined from this study.

The endogenous dehydrogenase enzyme activity of the heart was raised in the presence of hypertensive blood pressure levels. This appears to have some relationship to the degree of blood pressure attained. Conversely reserpine lowered this activity significantly. Reserpine has been shown to produce a reduction in amplitude and frequency of contractions in the isolated cat and rabbit hearts (13). Thus the dehydrogenase enzyme activity may be a reflection of the work being done by the heart. This would account for the increased enzyme activity of the hypertensive heart. Whether reserpine produces this reduced enzyme activity in the heart directly or indirectly necessitates further investigation. Reduced enzyme activity resulting in reduced cardiac amplitude and frequency of contractions could possibly contribute to the hypotensive action of this substance.

Summary. 1) The endogenous dehydrogenase enzyme activity, as measured by the tetrazolium technic, was determined in kidney, adrenal, thyroid, pituitary and heart tis-

sue from untreated and reserpine treated hypertensive animals. Experimental renal hypertension was produced in rats by 3 different methods. 2) A reduction in endogenous dehydrogenase enzyme activity of kidney tissue was observed in hypertensive animals while the activity of cardiac muscle was increased. Treatment with reserpine resulted in a depression of the endogenous dehydrogenase enzyme activity in cardiac tissue of both hypertensive and normal animals.

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Clotting of Hemophilic Blood with Purified Platelet Cofactor I, Platelet Factor 3 and Threone.* (23299)

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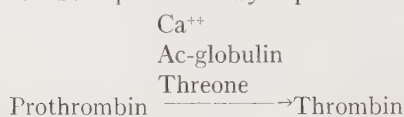
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When blood clots a large number of substances are involved in many chemical interactions. These are as numerous and complex as intracellular processes, and one way to

satisfy our curiosity about them is to study each chemical reaction separately. Before that can be done the substances involved must first be obtained in purified form. With that accomplished there is an additional opportunity in finding out what happens when such substances are added to blood as found in the

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veins of the average person or of one with a bleeding or thrombosing tendency. Two substances of particular interest in the clotting of blood are platelet factor 3 and platelet cofactor I. Both have recently been obtained in purified form(1,2). One is a lipoprotein of platelets and the other a plasma protein. The two together in solution, but still as unaltered independent entities, are by definition called threone(3). The activity is concerned with conversion of prothrombin to thrombin and can be represented by equation:



Threone activity is not found in serum even if platelet factor 3 is supplied. This is explained on the basis that platelet cofactor I combines with an inhibitor during the clotting of blood. Ether extraction presumably removes this inhibitor to set the platelet cofactor I free; for, ether extracted serum *does* have platelet cofactor I activity(4). The low threone activity of hemophilia A can also be increased by extracting the plasma with ether. This is consistent with the view that an inhibitor retards the function of platelet cofactor I in clotting of hemophilic blood. From this viewpoint we wondered what would be observed upon adding purified platelet cofactor I or purified platelet factor 3 to the whole blood of hemophiliacs.

Methods. Platelet factor 3: This was purified from bovine plasma and assayed as described by Alkjaersig, Abe and Seegers(1). *Platelet cofactor I:* This was purified from bovine plasma as described by Seegers, Landaburu and Fenichel(2). The preparation had a high activity and with the ultracentrifuge, products of this kind develop virtually a single boundary pattern. *Hemophilic blood and plasma:* Siliconed syringes and glassware were used throughout. Blood was drawn with 2 syringe technic. The plasma was obtained by centrifugation of blood mixed with 19% sodium citrate in the proportion of 49 to 1 respectively. The hemophiliacs were carefully studied in extensive laboratory tests and clinical observations and typed and graded in accordance with previously described criteria.

TABLE I. Clotting Time of Blood in Seconds at 37°C, and in Siliconed Tubes.

Patient	Whole blood	Whole blood plus—		
		Saline*	Co-factor I†	Platelet factor 3‡
Hemophilia A				
Grade 3	10,000	8,500	1,475	<410
" 1	7,150	5,050	1,575	195
" 2 to 3	20,000	20,000	620	185
" 2	12,600	9,600	1,075	270
Control	1,200	1,080	150	175

* .1 ml 0.9% NaCl plus 1 ml blood.

† Five mg platelet cofactor I dissolved in 1 ml 0.9% NaCl. Take 0.1 ml plus 1 ml blood.

‡ Purified platelet factor 3 in conc. of 720 units per ml. Take 0.1 ml plus 1 ml blood.

Grade 1 is the least severe disease, 2 and 3 more severe and 4 the most severe. *Clotting tests:* These tests are outlined in footnotes to the 2 tables.

Results. Whole blood clotting time is somewhat longer than blood diluted simply with saline (Table I). The clotting time was however, much shorter when the saline contained purified platelet cofactor I or purified platelet factor 3. Each of these factors added separately thus accelerates the clotting of normal blood. With all of the blood samples obtained from the hemophiliacs the whole blood clotting time was between 2 and 5 hours even on slight dilution with saline. With the addition of platelet cofactor I clotting time was approximately equal to that of normal whole blood, but not at all equal to that of normal blood to which platelet cofactor I had also been added. Purified platelet factor 3, in the selected concentration, was far more effective than platelet cofactor I. In fact, in certain instances, the clotting times were almost as low as with normal blood.

In the recalcified clotting time experiments (Table II) practically all of the results could have been predicted from the work with whole blood. The platelet factor 3 was most effective and purified platelet cofactor I reduced clotting time to normal; that is, to what the normal is without added cofactor I. One of the clotting times with cofactor I was quite long. A mixture of platelet cofactor I and platelet factor 3 was made so that the concentration of each would be exactly half of that used in other experiments. Thus the

TABLE II. Recalcified Plasma Clotting Times, in Seconds at 37°C, and in Siliconed Tubes.

Patient	Plasma only	Plasma* plus		
		Platelet factor 3†	Platelet cofactor I‡	Threone §
Hemophilia A				
Grade 3	>20 hr	98	1,500	141
" 1	"	98	1,530	144
" 2 to 3	"	97	1,090	128
" 2	"	115	6,700	205
Control	1,120 sec.	82	125	47

* .1 ml of .1 M CaCl_2 plus .5 ml plasma.

† In .1 M CaCl_2 platelet factor 3 in conc. of 360 units/ml. Take .1 ml of this platelet factor 3 solution plus .5 ml plasma.

‡ .5 mg platelet cofactor I in .1 ml of .1 M CaCl_2 plus .5 ml plasma.

§ In .1 M CaCl_2 360 units platelet factor 3/ml plus equal vol of 5 mg platelet cofactor I dissolved in .1 M CaCl_2 . Take .1 ml of this mixture plus .5 ml plasma.

concentration of each was reduced by 50%, but both were present simultaneously. Hence we had threone. The clotting time found was below that with platelet cofactor I alone, but not as low as with platelet factor 3. A comparatively low clotting time was observed with the normal plasma.

Discussion. Recalcified clotting times after addition of our purified platelet cofactor I, platelet factor 3 or with threone have not been measured previously. There are variations in the figures obtained, that arouse curiosity and it would be most interesting to extend a study of this kind to include numerous hemophiliacs and individuals with thrombosing tendencies. Quite possibly such simple empirical tests would uncover helpful correlations not detected by other tests. We can readily see that hemophilic blood and plasma are an-

tagonistic to cofactor I, platelet factor 3 and threone, in accord with the fact that in hemophilia A an inhibitor of the first phase of clotting is present in excess(5).

Perhaps occurrences in test tubes cannot serve as a basis for accurate predictions of therapeutic effectiveness *in vivo*. There are many substances (tissue thromboplastin, urine concentrates, fresh serum, thrombin) which when added to hemophilic blood will reduce its clotting time. These materials act probably by bypassing the fundamental defect, and the substances here described may well belong in this group. However, large quantities of platelet cofactor I given intravenously might be of help for short periods of time in hemophilia. Favorable results would be more likely with platelet factor 3 or with threone. In the purity and concentration used by us these are powerful coagulants.

Summary. Purified platelet cofactor I, purified platelet factor 3 or the two together reduce the clotting time of hemophilic blood or recalcified plasma; but, not to the degree observed with normal blood or plasma. In the concentrations used platelet factor 3 was more effective than platelet cofactor I.

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Resolution of Aortic Atherosclerotic Infiltration in the Rabbit by Phosphatide Infusion.* (23300)

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Earlier studies from this laboratory(1,2) have demonstrated that a *sustained* rise in the plasma phospholipid of either the rat or rabbit, induced by intravenous infusion of a suitable mixture of phosphatides, quickly leads to a hypercholesteremia in these species. A third study(3) also demonstrated that this phosphatide-induced hypercholesteremia could occur almost as well in the liverless as in the intact animal. This last finding of course indicated that the infused phosphatide was bringing into the blood, cholesterol from parts of the body other than the liver.

In view of these findings, it was thought advisable to determine whether a series of phosphatide infusions, administered to rabbits previously made hypercholesteremic and atherosclerotic by dietary ingestion of cholesterol, might alter the cholesterol content and extent of their aortic atherosclerotic process.

Methods. Two series of hypercholesteremic rabbits were studied. A very moderate hypercholesteremia was induced in the first series of 10 healthy male rabbits (avg age: 6 weeks; appx wt: 1500-1800 g) by placing them on Purina rabbit chow, containing 1% cholesterol and 2% cottonseed oil, for a period of 3 months. A very severe hypercholesteremia was induced in the second series of 14 rabbits by adding 3% cholesterol and 4% cottonseed oil to their Purina rabbit chow for a period of 3 months. At the end of this period, both series were returned to their regular rabbit chow diet. Plasma samples were taken before and then monthly after the high cholesterol feeding had been begun and analyzed for cholesterol(4). Monthly plasma samples also were obtained after the diet had been discontinued. Three months after cessation of excess cholesterol and oil feeding, the hypercholesteremia observed during the

cholesterol feeding had disappeared. At this time, for control purposes, 4 rabbits of the second series were sacrificed and their aortas were examined for gross atherosclerosis and a segment of it was analyzed for cholesterol. This segment (1 cm in length) was taken at a site uniformly 3 cm distant from the semi-lunar valves. For additional controls, the aortas of 5 completely normal rabbits were analyzed for total cholesterol content. The average plasma cholesterol of the remaining rabbits of both series was calculated for only the period of high cholesterol feeding by averaging the 4 samples obtained just prior to, and for 3 successive months, respectively, while they were on the special diet. These latter values then were employed to divide the rabbits of each series into pairs, of which each member had approximately the same degree of previous hypercholesteremia. After this was done, a plastic cannula (external diameter: 1.5 mm) was inserted under ether anesthesia into the external jugular vein of each rabbit. The catheter then was filled with saline, temporarily sealed at its open end and, by tunnelling beneath the skin of the neck, was brought out into the right external auditory canal. Folding of the ear with adhesive then protected the catheter from accidental displacement. Approximately one week after insertion of the cannula, 5 of the 10 rabbits of the first series and 5 of the 10 rabbits of the second series were placed in restraining cages and given rapidly via their catheter, 15 ml of a 4% suspension of phosphatides[†] in 5% dextrose, normal saline solution, followed by continuous infusion of the same fluid at a rate of 5 ml/hour for 10 hours. The remaining paired rabbits of each series were similarly treated except they received only the dextrose, normal saline infusions. The animals were rested for 48 hours and then the

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† "Lecithin (Animal) 90% Pure," purchased from Nutritional Biochemicals Corp., Cleveland, O.

TABLE I. Effect of Repeated Infusions of Phosphatide Suspension upon Atherosclerotic Infiltration and Cholesterol Content of Rabbit Aorta.

Procedure	No. of rabbits	Avg plasma cholesterol (mg/100 ml)		Aorta	
		During cholesterol feeding	Beginning of exp.	Atherosclerosis	Cholesterol (g/100 g)
Normal rabbits	5	—	64 ± 6 (52-84)	0	.4 ± .03 (.35-.44)
<i>Hypercholesteremic Series I</i>					
(a) Dextrose infused (control)	5	175 ± 14.2 (151-205)	72 ± 7.5 (65-92)	.8 (.5-1.5)	.60 ± .02 (.49-.72)
(b) Phosphatide infused	5	187 ± 15.6 (147-280)	76 ± 6.4 (66-88)	0	.40 ± .02 (.37-.55)
<i>Series II</i>					
(a) Not infused (control)	4	1119 ± 42 (821-1329)	72 ± 6.8 (58-82)	5	16.1 ± 1.2 (12.9-18.4)
(b) Dextrose infused (control)	5	942 ± 52 (759-1081)	68 ± 7.2 (62-78)	4.4 (4-5)	12.8 ± 2.9 (4.7-22.7)
(c) Phosphatide infused	5	1006 ± 61 (802-1270)	71 ± 6.2 (58-76)	1.4 (.5-3)	3.1 ± .91 (.8-6.2)

infusion was again repeated. All rabbits of the first series received 4, and those of the second series, 5 infusions. Plasma samples usually obtained before and at the end of each infusion were analyzed for total cholesterol and phospholipid(5). At the end of the series of infusions, each of the treated rabbits and its paired, untreated control were sacrificed. The entire aorta of each was assessed visually for degree of aortic atherosclerosis according to an arbitrary scale of 0-5. A grade of 1 was employed to describe an aorta having 20% of its total internal surface involved in the atherosclerotic process; a grade of 2, to describe an involvement of 40%, etc. Thus a grade of 5 was given to an aorta exhibiting almost total involvement. In addition, a segment of aorta was obtained, air dried, and analyzed for its total cholesterol content(6).

Results. The infusion of phosphatide was found, as previously reported(1,2), to elevate both the average plasma phospholipid and cholesterol. Thus in a typical infusion, the average plasma phospholipid of the rabbits of both series was raised from 110 to 750 mg/100 ml and the cholesterol from 58 to 120 mg/100 ml. This hypercholesteremia, however, was a temporary phenomenon and usually disappeared within the succeeding 48-hour period. The animals appeared to exhibit no ill effects from the repeated phos-

phatide infusions.

When the 5 treated rabbits of the first series and their paired controls were autopsied and their aortas examined, it was immediately apparent that in each pair, the treated rabbit exhibited no detectable atherosclerotic infiltration (Table I). However, 4 of the 5 paired rabbits receiving only dextrose-saline solution exhibited a slight but significant degree of infiltration in the arch of the aorta and about the orifices of the intercostal arteries. The cholesterol analyses of these aortas confirmed the gross findings (Table I) in that the aortas of the treated rabbits contained considerably less cholesterol than those of their paired controls. Indeed, in this first series no significant difference, either in gross appearance or in cholesterol content, was observed between the aortas of the treated animals and those of normal rabbits.

Similar changes were observed in the aortas of the treated rabbits of the second series. Whereas the group of 4 control rabbits sacrificed before the infusion and the group of 5 rabbits given only dextrose infusion both exhibited intense and widespread confluent atherosclerotic infiltration of the aorta, extending in most instances from the semilunar valves down to the bifurcation, the aortas of the 5 treated rabbits (infused with phosphatide suspension) exhibited only scattered and

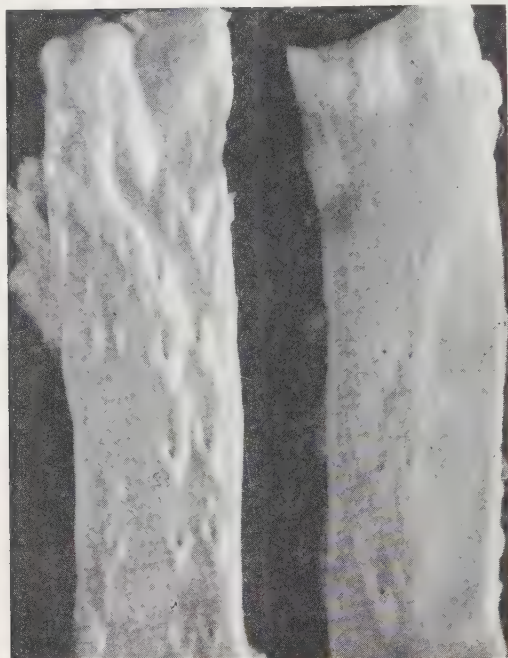


FIG. 1. Aortic segments of rabbit No. 912 (left) and rabbit No. 920 (right). Avg plasma cholesterol content of rabbit No. 912 during period of cholesterol feeding was 1174 mg/100 cc and that of rabbit No. 920 was 1159 mg/100 cc. Rabbit No. 920 received 5 infusions of phosphatide suspension. Rabbit No. 912 received only dextrose-saline infusion.

far less exuberant plaques (Fig. 1). As was expected from the gross findings, the average cholesterol content of the aortic segments of the control rabbits infused with dextrose-saline solution was 4 times as great as that found in comparable aortic segments of the rabbits treated with phosphatide.

Discussion. The difference observed in the present studies, between the aortas of previously hypercholesteremic rabbits given infusions of phosphatide suspension and those of the controls, was marked and striking. In the first series of rabbits made moderately hypercholesteremic, neither atherosclerotic infiltration nor excess cholesterol in the aorta could be detected after four infusions of phosphatide. However, 4 of the 5 paired controls exhibited some atherosclerosis and excess cholesterol in their aortas.

Perhaps even more dramatic changes were

observed in the second series of rabbits. These rabbits, previously made intensely hypercholesteremic and then infused with phosphatide, exhibited far less atherosclerotic infiltration and far less excess cholesterol in their aortas than was observed in the aortas of control rabbits of a similar degree of hypercholesteremia. This was true regardless of whether these latter rabbits were examined before the period of phosphatide infusions or after a comparable period of infusions with dextrose-normal saline solution. In view of these findings, infusions of suitable phosphatide suspensions appear to us to have possible therapeutic value.

Apparently, as we suggested in a preceding study(3), the *temporary* hypercholesteremia induced in our rabbits during the infusion of phosphatide represents a mobilization of cholesterol in plasma, derived in part at least from their aortic deposits of cholesterol. This excess cholesterol in turn probably was taken up by their livers(6), converted to bile acids (7) and hence excreted.

Summary. Intermittent intravenous infusion of a phosphatide emulsion into 2 series of previously hypercholesteremic rabbits appeared to effect a marked resolution of their atherosclerotic infiltration and cholesterol deposit as judged by the findings in paired, untreated animals.

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Ultra-Violet Damage to Living HeLa Cells as Recorded by Time-Lapse Motion Picture Studies.* (23301)

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In previous communications the authors have described the technics of ultra-violet flying spot television microscopy and of time-lapse motion picture photography for the recording of results(1,2,3). The purpose of this paper will be to report a preliminary study of several types of ultra-violet induced damage occurring in living HeLa cells when subjected to continuous scanning by the ultra-violet cathode ray tube.

Methods: The present cathode ray scanner tube has a peak emission at 2580 Å with a $\frac{1}{2}$ band width of 1000 Å. This spectral emission curve was further modified by inserting at the ocular of the microscope an interference filter. The spot scanning the specimen thus had a peak intensity at 2650 Å with a $\frac{1}{2}$ band width of 75 Å. The spot was made to sweep a 400-line/inch raster every 4 seconds and the resulting radar type monitor tube image was then recorded anew every 4 seconds on a single frame of 16 mm motion picture film. For our purposes, living HeLa cells were carried in continuous culture in a medium consisting of chick embryo extract, human ascitic fluid and Hanks balanced salt solution. During the study the cells were incubated at 37½°C on bare Vycor cover slip preparations which were in turn sealed to Vycor slides by a mixture of vaseline and paraffin. No differences in absorption were noted in cells mounted in balanced salt alone or in cells mounted in stock media. During the entire experiment the cells were continuously scanned and the results continuously recorded photographically.

Results. When the beam current of the scanner tube was adjusted to 10 micro-amperes, continuous photographic recording of the cells was possible for 6 to 10 hours with no detectable evidence of damage. Thus, for ex-

ample, 6 individual cells were observed to pass uninterrupted completely through their mitotic cycle, even though ultra-violet scanning was in some instances begun well before the prophase of mitosis took place. These cells required an average of about 30 minutes to complete mitosis. All of these cells showed typical surface bubbling of mitosis, and these small, rapidly appearing and disappearing bubbles contained cytoplasmic absorbing material. In addition, some of the cells showed 1 or 2 quite large non-absorbing bubbles. The lack of absorbing material in these large, more slowly appearing bubbles was attributed to syneresis. At this beam current typical intermitotic cells were spread out on the Vycor cover slip in an ameboid fashion. They showed continuous pinocytosis at their cytoplasmic borders and gradually increasing cytoplasmic absorption as the perinuclear area was approached. Clustered about the nucleus were small round droplets of lipid which were opaque to ultra-violet. In the undamaged cell these small black granules showed active Brownian motion during the entire course of observation. The nucleus of the intermitotic undamaged cell showed little or no absorption at 2650 Å except for absorption by the nucleoli.

When the beam current of the scanner tube was raised to 15 micro-amperes, cells appeared unable to complete mitosis. In this study cells were each observed for 96 minutes. Both cells were in early metaphase at the beginning of the observations. During the period of observation neither cell was capable of reaching the completion of mitosis. Both cells showed a marked diminution in the number of small bubbles containing cytoplasmic absorbing material, and a very marked increase in number of large non-absorbing bubbles. At any one time as many as 10 such non-absorbing bubbles were present in various places on the surface of each cell, and any one cell dis-

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played a total of 30 or more such bubbles during the period of observation. The marked increase in number of large non-absorbing bubbles, the decrease in number of small absorbing bubbles, and the failure of cells to complete mitosis during the time of observation were taken to indicate ultra-violet damage.

When single ameboid intermitotic cells were observed with the beam current of the scanner tube adjusted to 15 micro-amperes, they remained visually unaltered for the first 47 minutes. During the next 15 minutes there was a gradual slowing and then a complete cessation of Brownian motion of the lipid droplets. This was accompanied by a gradual cessation of pinocytosis at the cell borders. The cells then remained in this rather fixed state for the remainder of the 20-minute observation period. During this entire period no other visual changes occurred. In particular, there was no change in the absorption pattern of either the nucleus, nuclear membrane or cytoplasm. The cessation of pinocytosis and of Brownian motion of the fat droplets was considered to be due to a gelling of the cytoplasm as a manifestation of ultra-violet damage.

When the beam current of the scanner tube was adjusted to 25 micro-amperes, a different and more classical type of irradiation damage was shown by the intermitotic cell. In this study, a field of 14 cells was continuously scanned for 47 minutes. At the end of 10 minutes the first evidence of ultra-violet damage appeared in one cell. This consisted of a very rapid generalized increase in absorption of the cell. Two minutes later the entire cell was completely opaque to the ultra-violet light and at this time it contracted very suddenly into a small round black ball. The surface of this cell then showed some minor bubbling for the following few moments; and thereafter it remained as a small, round, completely opaque black ball. Throughout the remainder of the 37 minutes of observation, 9 other cells showed an identical sequence of events. These tended to occur as single events with one cell after another showing evidence of damage. At the end of 47 minutes of observation there were still 4 intermitotic cells

which showed no evidence of ultra-violet damage despite the fact that they were simultaneously scanned.

Discussion. Ultra-violet light of 2600 Å wave length has long been known to be extremely damaging to living material. The excellent studies of Bovie(4) in this regard demonstrated that marked changes in the ultra-violet absorption image of living amoebae occurred after only 15 seconds of irradiation. These changes consisted of a pronounced diminution of cytoplasmic absorption with an increase in nuclear absorption. He interpreted these findings to indicate a loss of the many colloidal interfaces in the cell such that formerly immiscible areas were rendered miscible with a resulting change in the absorption image. The changes described by Bovie are quite similar to the changes recorded in this experiment with the higher levels of irradiation.

Brumberg and Larionow(5) reported that the undamaged living cell showed a lack of nuclear absorption when photographed at 2600 Å. This observation was in marked contrast to the previous observations of Caspersson(6). Walker and Davies(7) were unable to confirm the results of Brumberg and Larionow, and concluded that the undamaged living cell did show nuclear absorption when photographed at 2600 Å. In some cases this nuclear absorption at 2600 Å exceeded the cytoplasmic absorption. Our own results indicate that the undamaged intermitotic HeLa cell nucleus, exclusive of the nucleoli, shows little or no ultra-violet absorption at 2650 Å. They further indicate that this condition obtains even after significant ultra-violet induced damage can be observed elsewhere in the cell upon examination of the motion picture film. This damage consists of a gelling of the cytoplasm with a cessation of Brownian motion of the lipid droplets and a cessation of pinocytosis.

Summary. 1. When living HeLa cells are continuously irradiated by ultra-violet emitting cathode ray scanner tube, no ultra-violet damage can be detected when low scanner tube beam currents are used. 2. When the beam current is increased, dividing cells show (a) inability to complete mitosis, (b) in-

creased syneresis during mitosis, and (c) decreased cytoplasmic bubbling during mitosis. 3. With a similar beam current, intermitotic cells show (a) cessation of pinocytosis, and (b) cessation of Brownian motion of cytoplasmic lipid droplets, even though no change occurs in the absorption image or the structural image of the cell. 4. When the beam current is further increased, intermitotic cells show a sudden generalized increase in absorption followed immediately by a marked contraction of the cell into a small round intensely absorbing mass.

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A Stable Preparation of Antigen-Sensitized Erythrocytes. (23302)

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In work reported elsewhere(1), the hemagglutination technic devised by Boyden(2) was used to assay the antibody-forming capacity of spleen cultures from rabbits given either bovine serum albumin (BSA) or bovine γ -globulin (BGG). This method, however, has the disadvantage of not making a stable preparation of antigen-sensitized erythrocytes, requiring preparation of freshly sensitized cells for each day's titrations. The present paper describes a method by which sensitized cells may be prepared which remain stable in the frozen or lyophilized state.

Methods. A 50% saline suspension of washed sheep red blood cells was treated with an equal volume of a 37% formaldehyde solution containing 0.9% NaCl for 2-3 days at 4°C. Since formaldehyde causes clumping of the cells, it was usually necessary to disperse these clumps by homogenization in a Waring blender for 1 hour at 2-5°C. After centrifuging, only the heavy cell layer was saved. No attempt was made to save the lighter material which separates to the surface as a pellet. The formaldehyde was removed by 7-8 washings in 5-6 volumes of saline over a ten-day period, followed by treatment of a 25% cell

suspension with 5% NaHSO₃ at 4°C overnight. The formaldehyde-bisulfite complex was then removed by dialysis against running tap water for 24 hours. Eegriwe's chromotropic acid test for formaldehyde(3) was used to detect residual formaldehyde. Unless all formaldehyde is removed, spontaneous agglutination occurs. A 50% suspension of the formalinized cells in saline was stable at 4°C for at least 5 months. The sensitization of the cells to the protein antigens after formalinization was essentially the same as the Boyden technic(2). However, when several liters of cells were prepared at one time, the times of treatment with tannic acid and antigens were extended to 30 minutes and one hour respectively. The sensitization was carried out as follows. One volume of a 2.5% suspension of formalinized cells was incubated at 37°C for 30 minutes with 1 volume of a 1:20,000 dilution of tannic acid (Merck, reagent grade) in 0.9% NaCl. The cells were washed once with 0.15 M phosphate buffered saline (PBS) at pH 7.3, centrifuged at 1500 rpm for 5 minutes and resuspended to volume in saline. One volume of these cells was incubated at room temperature for 1 hour

TABLE I. Hemagglutinin (HA) Titers of Anti-BGG Culture Fluids Using Normal Freshly Sensitized Cells, Formalinized Frozen Sensitized Cells, and the Latter Lyophilized.

Fluid No.	HA titer		
	Normal	Formal-inized	Lyophilized
1	8	16	16
2	8	4	8
3	4	8	16
4	8	8	8
5	16	32	64
6	16	8	8
7	8	16	16
8	32	32	64
9	16	32	64
10	8	32	32
11	32	64	64
12	64	128	128
13	16	32	32
14	8	8	8
Anti-BGG serum	12,800	25,600	12,800
<i>Idem</i>	25,600	12,800	ND*
"	25,600	25,600	ND
Normal rabbit serum	< 2	< 2	< 2

* Not done.

in 4 volumes of PBS, pH 6.4, containing 0.5 mg/ml of BGG (Armour's Fraction II) or the same amount of BSA (Armour's Fraction V) for each ml of cells to be sensitized. The cells were washed once with 1.0% normal rabbit serum (NRS) in 0.9% saline, centrifuged at 1500 rpm for 10 minutes and reconstituted to a 2.5% suspension in 1.0% NRS in 0.9% saline. These sensitized cells were then frozen at -70°C and stored at -40°C . Microscopically, the cells appear fairly normal and are impervious to hemolysis in hypotonic solutions. The cells are equally stable in the lyophilized state.

Results. Table I shows the results of titrating several tissue culture fluids from spleen cultures of rabbits given BGG intravenously(1). These were chosen at random from fluids frozen 6-8 weeks. A hyper-immune anti-BGG rabbit serum was included for comparison of titers. All titrations were carried out in serial 2-fold dilutions with 1:100 NRS in 0.9% NaCl as the diluent. Fifteen hundredths (0.15) ml of a 2.5% suspension of sensitized cells (about 6×10^{10} cells) was added to each tube, the racks shaken and left at room temperature for 2 hours. They were then placed in the cold

room and the HA end-points read the following morning. It is seen that the titers are within the range of dilution error (2 tubes) using freshly sensitized normal cells, frozen formalinized cells stored at -40°C up to 5 months, or the latter lyophilized and stored at room temperature for 6 months. Cells sensitized to casein or BSA and titrated with the homologous antiserum gave equally satisfactory results.

Discussion. Flick(4) treated human erythrocytes (group not specified) with formalin, and thus prepared a stable indicator useful for influenza A virus hemagglutination. Since these cells were unadaptable to the Salk pattern method(5) due to spontaneous agglutination, the turbidometric technic(6) was used. Cole and Farrell(7) described a method for coupling tuberculin PPD to formalinized Group A human red blood cells using tetrazotized benzidine. They then were able to titrate serum antibody to tuberculo-protein by an hemagglutination end-point pattern. Two attempts were made in this laboratory to couple BSA and BGG to sheep erythrocytes by this method, but spontaneous agglutination occurred both times. This may have been due to incomplete removal of the formaldehyde, or to a difference in the receptivity of sheep cells for proteins by this method.

When formalinized cells were sensitized to serum proteins and titrated against heterologous serum protein antisera, some cross reaction was seen. This is perhaps explained by the incomplete separation of serum proteins in fractionation. When cells were sensitized to purified casein (Difco) and titrated against anti-BSA or anti-BGG rabbit sera, no cross reaction occurred.

Summary. 1. A method has been described by which bovine serum albumin and bovine γ -globulin may be coupled to formalinized erythrocytes. 2. These sensitized cells are stable in either the frozen or lyophilized state for at least 5-6 months.

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Studies on Nonspecific Acquired Resistance to Viral Toxicity in Mice.* (23303)

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Results obtained in several laboratories have indicated that prior injection of viable or inactivated influenza virus protects mice to challenge with toxic quantities of virus administered by the same route. The rapidity with which resistance develops, and its production by serologically heterologous virus, preclude the possibility that specific immunity based upon antibody production is involved in such protection. Such protection has been produced in mice both by intracerebral(1,2) and intravenous(3,4) routes and resembles viral interference. On the other hand, susceptibility of experimental animals to bacterial infection or endotoxin has been modified by a number of nonspecific physiological factors(5-8). For example prior injection of bacterial endotoxin protected mice against subsequent bacterial infection or challenge with endotoxin, and was associated with an elevation of the properdin level of the serum (6-7). The present investigation was undertaken to determine what relation, if any, these phenomena might have to one another.

Methods. The PR8 and NWS (neurotropic) strains of influenza A virus, the Lee strain of influenza B virus, and the RO strain of Newcastle disease virus (NDV) were cultivated in the allantoic sac of embryonated

eggs. Virus was sedimented in a Spinco model L ultracentrifuge at 78,000 x g, resuspended in 1/10 the original volume of tryptose broth, and stored at -70°C. Techniques for demonstrating the toxic effects of intracerebrally injected influenza viruses(9,10) and NDV(11,12) have been described. Protection tests in mice were performed similar to that described by Wagner(1). Weanling Swiss albino mice weighing 9-10 g each were used for intracerebral inoculation. Prechallenge injections were 0.01 ml, while challenge injections made into the opposite hemisphere were 0.03 ml. All intravenous injections were 0.5 ml into the tail vein of mice weighing 18-20 g each. All mice were observed daily for 5 days and necropsied when the experiment was terminated. *Escherichia coli* endotoxin was prepared by the trichloroacetic acid method(13). The LD₅₀ of these preparations was approximately 0.18 mg when injected intravenously into 20 g mice.

Results. Table I summarizes the results of 3 experiments showing that prior intracerebral injection of a number of unrelated materials exerted a modifying effect on the neurotoxic effect of influenza A virus. Groups of 20 or 40 mice each were injected intracerebrally with the indicated amounts of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), purified type III pneumococcus polysaccharide, *E. coli* endotoxin, undiluted horse serum, or with saline. RNA and DNA were adjusted to pH 6.8-7.0 with NaOH and were used, as was the pneumococcus polysaccharide, at the limit of solubility. Twenty-four hours later, half of the mice in each

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[†] Data in this report were included in a Ph.D. thesis submitted to the Rutgers Graduate School in Feb. 1957. Present address: Dept. of Bacteriology, School of Medicine, University of Rochester.

TABLE I. Nonspecific Resistance to Neurotoxicity of Influenza A Virus in Mouse Brain.

Material injected	ED ₅₀ of challenge virus	
	2.7	5.4
-24 hr	C/T	
10 μ g RNA	1/10	5/10
10 μ g DNA	3/10	9/10
100 μ g Type III pneumococcus polysaccharide*	1/9	5/9
Horse serum	6/10	8/10
Saline	15/22	20/22
-24 hr		
10.0 μ g <i>E. coli</i> endotoxin	0/8	1/10
1.0 " "	2/10	1/10
.1 " "	0/10	3/10
.01 " "	0/10	9/10
Saline	11/12	12/13
-24 hr		
.1 μ g <i>E. coli</i> endotoxin	2/19	15/20
Saline	18/19	20/20
-6 hr		
.1 μ g <i>E. coli</i> endotoxin	3/19	8/20
Saline	17/20	16/16
+6 hr		
.1 μ g <i>E. coli</i> endotoxin	19/19	18/18
Saline	18/18	18/19

C/T = No. of mice convulsing/total.

ED₅₀ = 50% tonic convulsive dose.

-24 hr = 24 hr before virus.

+6 hr = 6 hr after virus.

* Kindly provided by Dr. Michael Heidelberger.

group were challenged intracerebrally with 2.7 ED₅₀ (50% tonic convulsive doses) of influenza A virus and the remaining mice were injected with 5.4 ED₅₀ of virus. Groups of mice were also treated with endotoxin at 24 and 6 hours before and 6 hours after challenge with lethal doses of influenza virus. Twice daily for 3 days following challenge, each mouse was suspended by its tail and twirled vigorously to induce convulsions. Each mouse that convulsed was killed, and the total number of mice convulsing was recorded. The results show that large doses of RNA, DNA, and pneumococcus polysaccharide afforded partial protection against 2.7 or more ED₅₀ of influenza neurotoxin and that horse serum was without effect. *E. coli* endotoxin was most effective in protecting mice from the neurotoxicity of influenza virus. Indeed, as little as 0.01 μ g of endotoxin protected mice against 2.7 ED₅₀ of virus. Endotoxin was effective when given 6 or 24 hours

before virus, but afforded no protection when given 6 hours after challenge.

It has been shown(12) that intracerebral injection of the RO strain of NDV in high dilution into mice causes paralysis and death in the absence of detectable viral synthesis. Prior intracerebral injection of influenza B virus or receptor destroying enzyme (RDE) protected mice against the toxic effect of highly diluted virus and delayed onset of symptoms following injection of concentrated virus. Heat-inactivated RDE delayed response to concentrated NDV, but had no effect on frequency or time to paralysis or death induced by high dilutions of NDV. Wagner (1) found that prior intracerebral injection of RDE or sublethal doses of influenza A virus or influenza B virus protected mice from intracerebral infection with the neurotropic NWS strain of influenza A virus. The effect of *E. coli* endotoxin on the response of mice to NDV and the NWS strain of influenza virus injected intracerebrally is shown in Table II. In 2 experiments, groups of 20 mice each were injected intracerebrally with appropriate quantities of endotoxin or with saline. Twenty-four hours later, half of the mice in each group were injected intracerebrally with 10x concentrated NDV (10⁺¹ dilution) and the remainder were injected with NDV diluted 10⁻³. Each mouse was examined daily, and the time of onset of the first unmistakable response (paralysis or prostration) or death was recorded. In a separate experiment, groups of 10 mice each were injected with dilutions of endotoxin or saline and each group was challenged 24 hours later with approximately 100 LD₅₀ of NWS influenza virus. Mortality ratios were recorded and the average day of death was calculated for each group. The results show that prior injection of 0.001 μ g of endotoxin more than doubled the latent period in mice challenged with concentrated NDV, but 100 μ g of endotoxin had no effect on the response of mice to diluted NDV. Further, 10 μ g of endotoxin failed to influence infection with NWS influenza virus.

Recently, Khoobyarian and Walker(3) showed that mice were protected from intra-

TABLE II. Effect of *E. coli* Endotoxin on NDV and Neurotropic Influenza (NWS) Virus in Mouse Brain.

1st inj.* <i>E. coli</i> endotoxin inj. intracrer. (μ g)	2nd inj.				NWS 10 ⁻²
	RO-NDV				
	10 ⁺¹		10 ⁻³		
	Exp. A	Exp. B	Exp. A	Exp. B	
	ADR				
100.	3.9		5.3	5.2	
10.	3.9		5.9	5.9	6.9
1.	3.1		5.7	5.9	7.2
.1	3.4	3.5	6.3	6.4	7.1
.01	4.7	3.3	4.9	5.0	6.7
.001		3.0			
.0001		2.1			
Saline	1.5	1.4	6.4	6.0	6.5

* 10 mice/group.

ADR = Avg day of response. 10⁺¹ = 10 \times concentrated RO-NDV. NWS = NWS strain of influenza A virus.

venous toxicity of influenza virus by a prior intravenous injection of influenza A virus, influenza B virus, NDV, RDE, or heat-inactivated RDE. Prior injection of typhus rickettsiae, feline pneumonitis virus, or bacterial pyrogen in the form of typhoid vaccine were without effect. The data summarized in Table III show that a prior intravenous injection of *E. coli* endotoxin exerted a protective effect against the intravenous toxicity of influenza A virus. Groups of 20 or 40 mice each were injected intravenously with dilutions of influenza A virus, influenza B virus, NDV, *E. coli* endotoxin, or saline. Twenty-four hours later, groups of approximately 20 mice each were injected intravenously with 3 LD₅₀ of influenza A virus or 1.4 LD₅₀ of

E. coli endotoxin. The mortality ratios of mice dying within 5 days without pulmonary consolidation were recorded for each group. It is clear that prior injection of endotoxin induced tolerance to challenge with lethal doses of influenza virus and endotoxin. However, prior injection of influenza A virus failed to protect mice against endotoxin. As anticipated (3,4), influenza A virus, influenza B virus, NDV, and RDE protected mice against the toxicity of influenza A virus.

Extensive experiments, designed to demonstrate virus neutralizing factors in serum of mice that had received a protective dose of influenza A virus 24 hours previously, were unsuccessful. Further, prior intravenous injection of India ink and thorium dioxide failed to increase the resistance of mice to the toxic effects of influenza virus when barely tolerated amounts were administered 24 hours before challenge.

Discussion. The data presented confirm the fact that prior intravenous injection of sublethal quantities of bacterial endotoxin or influenza virus protected mice against subsequent challenge with the homologous agent. It is of particular interest that prior injection of endotoxin protected mice against subsequent inoculation of toxic quantities of influenza virus, while previous injection of influenza virus failed to protect mice against challenge with endotoxin administered by the same route. It would appear that the physiological mechanisms set into motion by intra-

TABLE III. Nonspecific Resistance to Viral Toxicity Produced in Mice by Intravenous Injection of *E. coli* Endotoxin.

1st inj.	2nd inj.	
	Influenza A virus	.25 mg endotoxin
	D/T	
Endotoxin, .1 mg	3/14	0/17
.08 "	4/16	
Saline	17/20	17/18
Influenza A virus: 10 ⁰	2/19	17/17
10 ⁻¹	0/20	
10 ⁻²	14/20	
Saline	18/20	11/20
Influenza B virus, 10 ⁰	0/20	
Newcastle disease virus, 10 ⁰	1/20	
Saline	37/37	

D/T = No. dead/total.

venous injection of endotoxin were capable of protecting mice against challenge with both endotoxin and toxic quantities of influenza virus, while prior intravenous injection of influenza virus failed to elicit such a response. It should be emphasized that barely tolerated quantities of endotoxin were necessary to induce tolerance to either endotoxin or influenza virus. This fact may account for the recently reported(3) failure of typhoid vaccine to protect mice against the toxicity of influenza virus.

It is of interest that prior intracerebral injection of minute quantities of endotoxin (Table I), as well as sublethal quantities of virus(1,2), exerted a protective effect in mice challenged 24 hours later with toxic quantities of influenza virus by the same route, while endotoxin failed to protect mice against infection with the neurotropic NWS strain of influenza virus (Table II). Since prior intracerebral injection of non-neurotropic influenza virus has been shown to interfere with the neurotropic strain in mouse brain(1) and with the neuropathic effect of dilute RO-NDV(12) failure of endotoxin under similar conditions to induce resistance suggests that the mechanisms of viral interference and acquired resistance to viral toxicity are different. It is of interest in this connection to recall that Wagner(14) was unable to demonstrate an increase in the resistance of HeLa cells in tissue culture to the cytotoxic action of influenza virus after exposure of the cells to subtoxic quantities of the virus.

Summary. 1) Prior intravenous injection of *Escherichia coli* endotoxin or influenza virus protected mice against subsequent challenge with the homologous agent. Prior intravenous injection of endotoxin protected mice against subsequent inoculation of toxic

quantities of influenza virus, while previous injection of influenza virus failed to protect mice against challenge with endotoxin administered by the same route. 2) Prior intracerebral injection of minute quantities of endotoxin, as well as sublethal quantities of influenza virus, exerted a protective effect in mice challenged 24 hours later with toxic quantities of influenza virus by the same route. Endotoxin failed to protect mice against infection with the neurotropic strain (NWS) of influenza virus or against the neuropathic effect of dilute Newcastle disease virus (RO strain). It is suggested that the mechanisms of viral interference and acquired resistance to viral toxicity are different.

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Coxsackie A7 Virus and the Russian "Poliovirus Type 4". (23304)

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In February 1956, Drs. M. P. Chumakov and M. K. Voroshilova from the Poliomyelitis Research Institute in Moscow made available to us their "Type 4 poliovirus." Details of the isolation of this virus in 1952 and its characteristics were subsequently published by these authors(1). In summary, 3 strains of a virus had been isolated from the stools of Russian children during the acute phase of a clinical illness characteristic of paralytic poliomyelitis. Strain AB IV, the strain supplied to our laboratory, was isolated by inoculation of monkeys with an admixture of stools from 2 patients with bulbar polio, which subsequently proved to be fatal. The virus caused paralysis and typical histological lesions of poliomyelitis after intramuscular (I.M.) or intracerebral (I.C.) inoculation of monkeys. It also caused paralysis and death in suckling mice and in both suckling and adult cotton rats. This virus was not neutralized by antisera against Types 1, 2, or 3 polioviruses, and showed no cross immunization with these viruses on I.C. challenge. It was reported to be cytopathogenic for human fibroblasts in tissue culture (T.C.) but to have low titer and with a 10 to 12-day incubation period.

The Russian virus was supplied to us in the form of glycerinated brains of the 24th suckling cotton rat passage, and no antiserum was made available at that time. This report details the studies conducted with the AB IV strain of the Russian virus, which have shown it to be identical with Coxsackie A7 virus and confirmed its properties as reported by the Russian investigators. Also reported here are studies on 2 strains of Coxsackie A7 virus isolated in the United States, and evidence is presented to indicate that these strains, like those of Chumakov and Voroshilova, are capable of producing in the central nervous

system (CNS) of some monkeys lesions which are indistinguishable from those caused by polioviruses.

Methods. Animal inoculation. Suckling Swiss mice less than 24 hours old were inoculated either intraperitoneally (I.P.) or I.C. with 0.03 and 0.02 ml respectively. Either brain or whole carcass was harvested for passage material. Rhesus monkeys were inoculated with 1.0 ml, either I.C. into the thalamic area or I.M. in the thigh. *Tissue culture.* Freshly prepared monkey kidney roller tube cultures (MKTC) grown and maintained in lactalbumin-calf serum-Hanks' solution medium were inoculated with 0.1 ml of original materials and passages were made with 0.1 ml of undiluted culture supernates. *Neutralization tests.* Routinely 100 infectious doses of virus were mixed with equal volumes of antiserum dilutions, incubated for 1 hour at room temperature and then inoculated into either 2 T.C. tubes or 8 suckling mice. Tubes were examined daily for 10 to 14 days, and mice for 14 days. *Pathology.* The methods used in preparation of the CNS of test monkeys for histological examination, and the procedure for sectioning and examination, were those recommended by the Technical Committee on Poliomyelitis Vaccine(2).

Results with Russian AB IV Strain. The original 24th passage suckling cotton rat brains were washed free of glycerin and emulsified at 10% concentration and used as inoculum for monkeys, suckling mice and tissue culture.

Suckling mice developed paralysis of the extremities after a 4 to 5-day incubation period and usually died in the subsequent 24 hours. The appearance of the mice was typical of Coxsackie A infection. On subsequent passages from these mice it was found that both brain and carcass had a titer of 10^5 by either I.P. or I.C. inoculation. This passage virus caused no symptoms when inoculated I.C. or intraspinally (I.S.) into adult mice.

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One *rhesus monkey* (73542) inoculated I.C. with original cotton rat virus, developed a slight elevation of temperature on the third to seventh days and moderate generalized tremors on the fourth day which persisted for one week. This monkey was bled on the 28th day, then given 5 ml of 20% suckling mouse brain passage 2 virus intramuscularly and bled on day 35, at which time it was killed for pathological examination. A second *rhesus monkey* (72929) received 1.0 ml of original virus I.M. in the right thigh. On the 3rd day it developed fever; tremors on the 4th day; and definite weakness of both legs on the 5th day. The muscle weakness had not progressed by the next day, so the animal was sacrificed. An emulsion of the lumbar cord of this monkey produced typical disease in suckling mice. An attempt to infect 5 *rhesus monkeys* by I.M. inoculation with this cord suspension (1 ml of 20% suspension) was clinically negative. No attempt was made to pass it intracerebrally in monkeys. The histological findings in both these monkeys were typical of polio, but in one only the spinal cord was involved.

Tissue cultures, including HeLa, human skin epithelium, human amnion, human fibroblasts and monkey kidney (MK) epithelial cells, were inoculated with the original cotton rat brain virus. There was no apparent cytopathogenic effect (CPE) except in MKTC. Here, after an incubation period of 8 to 10 days, a few scattered individual cells were seen to be round and refractile. Subsequent holding of the cultures did not result in any extension of this effect to the remainder of the cell sheet. The same results were obtained when the emulsified carcasses of the first suckling mouse passage were inoculated into MKTC. Subsequent passage of these materials again gave very few scattered cells showing CPE until the 7th passage. At this time the line established from suckling mouse virus showed widespread CPE after an incubation of 3 days. This T.C. line of AB IV virus has been carried through 33 passages and now titers to 10^6 , and with heavy inocula produces CPE within 24 hours. Passage 22, with a titer of 10^5 , was shown to produce CPE in HeLa, human amnion, and human fibroblast cells, but not in human skin cul-

tures. MKTC passage 8 was positive on I.C. inoculation into suckling mice, but passage 27 was negative.

Characteristics of the virus were investigated using the suckling mouse passage strain and it was found to be ether resistant, inactivated by 60°C for 10 minutes, filterable through a Seitz EK pad and a Selas 03, and not affected by penicillin, streptomycin or achromycin. It was negative on attempted passage in chicken embryos.

Identification of the Russian AB IV virus was attempted by neutralization, complement fixation and hemagglutination-inhibition tests. Since no specific antiserum had been supplied with the original virus strain, the only antiserum available during the primary investigations was that from the monkey convalescing from I.C. inoculation of the original cotton rat virus after receiving a booster dose of suckling mouse passage virus. This serum neutralized 100 LD₅₀ of suckling mouse virus at a 1/25 dilution in mice and 100 TCID₅₀ at a 1/25 dilution in MKTC. The serum was tested in neutralization tests in suckling and adult mice and in MKTC against the viruses indicated in Table I. It was negative against Types 1, 2, and 3 polioviruses and all others tested except Cocksackie A7 and A8. This same monkey antiserum was also tested by complement fixation and hemagglutination-inhibition against the viral antigens shown in Table I with negative results.[‡]

At a later date we obtained a sample of AB IV antiserum from the Russian virologists in the form of immune monkey serum. This was shown to neutralize our suckling mouse virus at a 1/625 dilution and the T.C. virus at 1/25. It also neutralized standard A7 virus in suckling mice.

The AB IV suckling mouse virus was tested for neutralization by the standard antisera listed in Table I with negative results except for Cocksackie A7 serum and normal human gamma globulin produced in the U.S.

Follow-up neutralizations, in view of the positive results with our AB IV antiserum

[‡] The hemagglutination-inhibition tests were kindly performed by Dr. Max Theiler of the Rockefeller Foundation Virus Laboratory and Dr. Leon Rosen of the N.I.H.

TABLE I. Immunological Tests Made to Identify Russian "Type 4 Poliovirus."

AB IV virus (SM) <i>vs</i> immune sera in SM	AB IV immune monkey serum <i>vs</i> viruses in—			
	Adult or suckling mice	MK tissue culture	Complement fixation	Hemagglutina- tion inhibition
Coxsackie A1, 2, 3, 4, 5, 6, 7,* 8, 9, 10	Mouse encephalo- myelitis-TO, FA	Herpes Vaccinia	Polio 1, 2 Coxsackie A10	Dengue 1, 2 West Nile
Coxsackie B1, 2, 3, 4, 5	GD VII	Echo 1, 2, 3, 4, 5, 6, 7, 8, 9, 10,	Adeno 7	Yellow fever
Mengo	Coxsackie A1, 2,	11, 12, 13, 14	Mumps	WEE
EMC	3, 4, 5, 6, 7,*	Polio 1, 2, 3		EEE
LCM	8,* 9, 10			Ilheus
St. Louis encephalitis				St. Louis enceph.
Polio 1, 2, 3				
Human gamma globulin A*				
" " " B*				

All negative except those starred. All neutralization tests with undiluted sera against 100 infectious doses of viruses.

against Coxsackie A7 and A8 viruses, showed that both the suckling mouse and T.C. viruses were neutralized by standard A7 immune mouse serum, but not by A8 antiserum. This seemed to establish the fact that the AB IV virus which we had isolated from the original cotton rat material by passage in both suckling mice and MKTC contained Coxsackie A7 virus. In view of the original isolation by the Russian investigators having been made from "paralytic polio" patients and the typical polio lesions produced in monkeys, we felt that further work was necessary to rule out an admixture of Coxsackie A7 with some other virus, especially a poliovirus.

Our monkey antiserum was shown to be negative against polioviruses 1, 2, and 3, and the AB IV virus negative in mice and T.C. against a mixture of antisera against these 3 viruses. The original cotton rat virus supplied by the Russian investigators as well as the monkey, T.C., and suckling mouse passages of it were completely neutralized by standard Coxsackie A7 antiserum. Furthermore, the 4th suckling mouse passage and 26th T.C. passage viruses were carried through 5 ultimate dilution passages and then shown to be neutralized with A7 antiserum. Antisera were produced in adult mice against these 2 ultimate dilution passage viruses and were shown to cross-neutralize each other in the 2 test systems, and were also positive against standard A7 virus in mice. The 5th ultimate dilution suckling mouse passage and the 23rd MKTC passage viruses were sent to Dr. Albert Sabin who kindly tested them for monkey pathogenicity by intraspinal inocula-

tion. Dr. Sabin reports that although no definite clinical paralysis developed in these animals, they showed poliomyelitis-like lesions with a distribution different from poliovirus infection.

Results with U.S. strains of Coxsackie A7. Frozen stool emulsions collected by Dr. Robert Parrott, formerly of N.I.H., from 2 children with asptic meningitis in 1952 and reported in our studies of nonparalytic poliomyelitis(3) as positive for Coxsackie A7 virus, were available for study. These same stool specimens had been tested by us in MKTC in 1954 with negative results, and Dr. Parrott had isolated A7 virus from them in 1952(4). The stool emulsions were again shown to contain A7 virus by inoculation of suckling mice. Each was also inoculated I.C. into a rhesus monkey. One monkey remained afebrile and showed no symptoms (79287), while the other (79286) had a temperature elevation from the 2nd to the 6th day, during which time there appeared some reluctance to use the left arm, but no definite weakness could be demonstrated. These monkeys were observed for 35 days when they were killed for histological

TABLE II. Results of Neutralization Tests with Sera of Different Age Groups against AB IV Virus (Coxsackie A7).

Ages (yr)	Neutralization test results	
	No. tested	No. positive
1-5	10	6
5-10	12	7
10-15	7	3

1/4 dilution of sera against 100 LD₅₀ virus in suckling mice. Sera from children in Washington, D. C. area.

TABLE III. Degree of Involvement and Distribution of CNS Lesions in Infected Monkeys.*

Monkey No.	Par. and NP polio†	72929	73542	80951	79286	79287	79900	79901
Inoculated with Day after inoc.	Poliovirus	AB IV, IM 6	AB IV, IC 35	Stool 6509, IC 7	Stool 6509, IC 35	Stool 6617, IC 35	Stool 6509, IC 37	Stool 6617, IC 37
Clinical signs		Tremors, fever, weak legs	Tremors, fever	Fever, weak arm	Fever, weak arm	None	Fever, ? tremors	Fever, ? weak arm
<i>Regions of CNS</i>								
Spinal grey	+	++(+)	+	++(+)	++(+)	++(+)	++(+)	++(+)
Cranial nerve nuc.	+	+++	0	++(+)	+++	++	++	++
Retie. formation	+	+++	0	++	++	++	++	++
Midbrain and subthal.	+	+++	0	++(+)	++	++(+)	++	++
Substantia nigra	+	+++	0	++	++(+)	++	++	++
Hypothalamus	+	+++	0	+++	++	++	++	++
Dorsal thalamus	+	++	0	++	++	++	++	++
Cerebellar areas:								
Cortex	+	0	0	0	++	0	0	0
Roof nuclei	+	++	0	++	+++	++	++	++
Dentate nucleus	+	++	0	++	++	++	++	++
Pontine nuclei	±	++	0	++	++	++	++	++
Corpus striatum:								
Caudate	0	0	0	0	++	0	0	0
Putamen	0	0	0	0	++	0	0	0
Amygdala	+	0	0	0	++	++	++	++
Pallidum	+	0	0	±	++	0	++	++
Olfactory centers	±				++	0	0	0
Cerebral cortex:								
Frontal	±	±	0	0	++	++	++	0
Pre-central	+	+	0	+	++	++	++	0
Post-central	+	0	0	0	++	++	++	0
Parietal	0	0	0	0	++	++	++	0
Temporal	0	0	0	0	++	++	++	0
Occipital	0	0	0	0	++	++	++	0
Hippocampus	0	0	0	0	++	++	++	0
White matter	±	+	0	±	++	++	++	0
Meninges	+	+	0	++	++	++	++	0

* Symbols: ++(+ + +) = Range of ++ to 4+; 0, no lesions; ±, absent or minimal lesions; +, mild or minimal inflammatory or glial infiltration; ++, moderate infiltration and minimal neuronal damage; ++++, moderate infiltration and moderate neuronal damage; ++++, severe infiltration and neuronal damage.

† From Table II, collection of D. Bodian, *Am. J. Hyg.*, v64, 104, 1956, Technical Committee on Poliomyelitis Vaccine and Sub-committee on the Monkey Safety Test.

examination of the CNS. Sera obtained before inoculation and at time of sacrifice were checked for antibodies against each of the 3 types of poliovirus with negative results and were shown to have developed neutralizing antibodies against our T.C. strain of AB IV (Coxsackie A7) virus. Typical polio lesions were found in the CNS of these 2 animals, but as indicated in Table III there were also lesions in "non-polio" areas. The first suckling mouse passage of the virus from the same 2 patients' stools were also each inoculated I.C. into a single rhesus monkey (79900 and 79901) with identical clinical and histopathological results except that the latter had lesions completely indistinguishable from those of poliomyelitis. Monkey 80951 was inoculated I.C. with one of the original stool suspensions and became febrile on the 5th day with slight weakness of the left arm, and was sacrificed on the 7th day. The CNS lesions were identical with severe poliomyelitis. An emulsion of brain stem titrated 10^2 in suckling mice and the cord to 10^4 and both were neutralized by Coxsackie A7 serum. The cord suspension was negative in MKTC.

Results of serum survey for Coxsackie A7 antibodies. Sera from 29 normal children of different ages in the Washington, D.C. area (ages 1 to 15 years) were made available by Dr. Robert Huebner of N.I.H. These sera at a $\frac{1}{4}$ dilution were tested against 100 LD₅₀ of AB IV (Coxsackie A7) suckling mouse passage virus in mice. The results summarized in Table II show that at each age level a good proportion of the sera possessed specific neutralizing antibodies against Coxsackie A7 virus. It had previously been found that one batch of U.S. produced gamma globulin had a neutralizing titer of 1/5 and another of 1/25 against this virus.

Pathologic picture in CNS of infected monkeys. The histopathological results summarized here are based on the detailed examination of the CNS tissues of 7 monkeys. Two monkeys (72929 and 80951) killed on the 7th postinoculation day showed lesions predominately in the anterior and lateral columns of the spinal cord. Degrees of neuronal degeneration varying from chromatolysis to vacuolization of cytoplasm, nuclear pyknosis

and neuronophagia were seen. Additional lesions were focal gliosis of grey matter accompanied by polymorphonuclear leukocytic and lymphocytic perivascular infiltrations. The remaining 5 monkeys (73542, 79286, 79287, 79900 and 79901) were killed between 30-35 days postinoculation, and presented lesions of a sub-acute type. The lesions were comprised of glial foci and lymphocytic perivascular infiltrations in grey matter. There was some loss of neurons in the spinal cord. Inflammatory and glial lesions occurred also in white matter. The intensity and distribution of lesions are shown in Table III.

The qualitative aspects of the pathologic lesions seen in sections of the CNS of these monkeys were not different from those seen in poliovirus infected animals nor from those suffering infection with some other neurotropic viruses. No inclusion bodies were demonstrated.

The distribution of lesions within the various areas of the CNS has been emphasized as the important differentiating criterion in determining whether a particular animal shows histological evidence of poliovirus infection (2). The distribution pattern of lesions in the 7 experimental monkeys is summarized in Table III, and for comparison purposes, that considered typical of poliovirus infection is also included. In summary, the degree of involvement and the distribution of lesions in 3 monkeys were identical to what might be seen in poliovirus infected monkeys. These include monkey 72929 killed in the acute stage of infection with the Russian strain, 79901 convalescent after receiving suckling mouse passage of one of the American strains, and 80951 killed in the acute stage after being infected by inoculation with human stool virus (Fig. 1).

The lesions confined to the spinal cord of monkey 73542 were qualitatively indistinguishable from those of poliomyelitis but not enough areas of the CNS were involved for distribution of lesions to be used as a differentiating characteristic. However, in 3 monkeys (79286, 79287, and 79900) the lesions were distributed in such a manner that permitted differentiation of their infections from those caused by polioviruses. The caudate

nucleus, putamen, or both, of these 3 animals showed lesions (Fig. 2 and 3). Additionally, 2 of the animals had definite though minimal

lesions of hippocampal and occipital cortex. These areas are rarely or never involved in poliovirus infection. The approximately

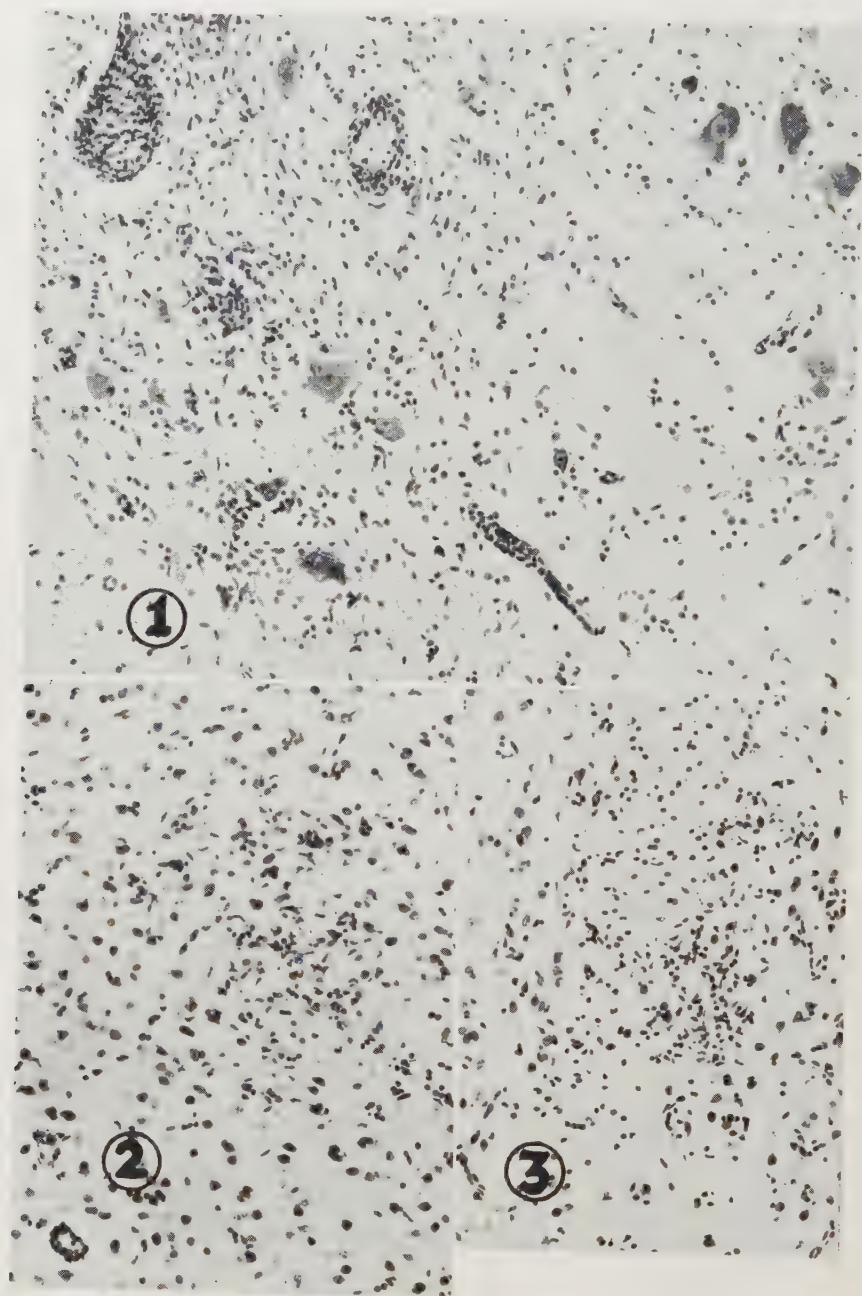


FIG. 1. Lesions caused by Coxsackie A7 in anterior horn of lumbar spinal cord. Perivascular infiltration, focal gliosis, chromatolysis and neuronophagia are illustrated. Monkey No. 80951, Galloeyanine stain, 143 \times .

FIG. 2. A glial focus in caudate nucleus of Monkey No. 79286, Gallo, 143 \times .

FIG. 3. A glial focus in putamen of Monkey No. 79286, Gallo, 143 \times .

equal degree of involvement of the motor (precentral) and of the anterior parietal (postcentral) cortex also was helpful in the differentiation.

Pathological examination of a few infected suckling mice showed typical Coxsackie A type myositis and no involvement of the pancreas or interscapular fat. In 2 suckling mice slight CNS involvement was observed, composed of sparse perivascular cuffing and small glial patches in cerebral cortex.

Discussion. The possibility that viral agents other than the 3 known types of poliovirus may cause a clinical illness in human beings similar to poliomyelitis has by now been substantiated insofar as nonparalytic polio is concerned. A number of agents have been shown to cause an aseptic meningitis clinically indistinguishable from nonparalytic polio(3,5,6,7).

The evidence for the etiological association of Coxsackie B types with the aseptic meningitis or nonparalytic poliomyelitis syndrome is quite well established(6,8,9) whereas for the A group the evidence is only suggestive(8,10). The high frequency with which group A types are isolated from children with minor illnesses or in good health makes their presence in poliomyelitis cases difficult to evaluate unless large numbers of proper controls are tested. In such a controlled experiment (3) we recovered A9 virus 5 times more frequently in the "nonparalytic poliomyelitis" patient group than the expected rate calculated on the basis of overall incidence in this group and 3 control groups of children.

In this same study Coxsackie A7 virus was isolated from the stools of 6 children hospitalized as nonparalytic poliomyelitis patients in Washington, D.C. in 1952. These isolations were made by inoculation of suckling mice by Dr. Robert Parrott, whereas the same stool specimens when tested by us in MKTC were negative. However, from different stool specimens on 2 of these cases we isolated type 2 polio and Echo 6 viruses in T.C. In the case of 4 of these 6 children, the A7 isolation had been made on specimens collected more than 7 days after admission to the hospital ward, during which time other patients with A7 in their stools were present on the same ward.

On this basis these 4 whose admission stools had been negative were considered to be hospital infections. The other 2 A7 cases could not be so explained. Johnsson(11) had 6 isolations of A7 virus in suckling mice inoculated with stools from 354 cases of aseptic meningitis, and Svedmyr at the Central Bacteriological Laboratory of Stockholm has isolated 2 strains of A7 in T.C. from children with aseptic meningitis. Stanley and Dorman(12) have described a virus isolated from the cord of a fatal case of paralytic poliomyelitis which is pathogenic for monkeys and suckling mice. The virus was not identified, but did not produce myositis and was not types 1 or 2 poliovirus.

In large-scale surveys of stools of children by suckling mouse inoculation, A7 isolations have been relatively few compared to other A types, yet the presence of specific antibodies in gamma globulin and the high incidence of positive neutralization tests with sera of young children found in the present study indicate that infection in the U.S. with this virus is probably a common experience during childhood.

In contrast to the ever-growing number of viruses capable of producing the nonparalytic poliomyelitis syndrome in humans, there are no known viruses, other than polioviruses, which have definitely been proven capable of causing the paralytic form of the disease in man. Yet as more complete investigations of paralytic poliomyelitis cases are carried out on larger numbers of individuals there will probably be more instances reported where poliovirus cannot be isolated from the stools and paired serum samples show no poliovirus antibody rise. Such paralytic cases have already been found(13,14). Likewise, except for the pathological findings in the Coxsackie A7 infected monkeys reported in this paper which confirm the Russian observations, no other human viruses have been found to cause histological lesions whose nature and distribution in the CNS of some infected monkeys are indistinguishable from those produced by polioviruses(2).

Chumakov *et al.*, have reported the isolation of a so-called "type 4 poliovirus" on the basis that the source was stools of acutely

paralysed children; the virus caused paralysis in monkeys with typical poliomyelitis lesions and antibodies were shown to develop in the blood of convalescent paralytic cases. The virus was shown to be immunologically distinct from the 3 known types of poliovirus. This "type 4 poliovirus" isolated by the Russian workers has now been shown to be a Cocksackie A7 strain in our laboratory and also by Dr. Torsten Johnsson in the State Bacteriological Laboratory at Stockholm. The mistaken identification by the Russian workers is understandable since they did not have specific Cocksackie virus typing sera available at the time.

We have confirmed the results of the Russian workers insofar as demonstrating that their A7 virus is capable of producing polio-like lesions in the monkey CNS and we have further demonstrated this to be true of 2 strains of A7 virus isolated directly from stools of children with aseptic meningitis in the U.S. Actually, on the basis of criteria established by 2 groups of poliomyelitis research workers(15,16), the A7 Cocksackie virus might well be considered a poliovirus. It has been isolated from cases of "nonparalytic" and "paralytic poliomyelitis"; it causes clinical and histological signs in monkeys consistent with poliomyelitis; it has physical properties similar to those of polioviruses; the range of host susceptibility is the same; and pooled human sera contain neutralizing antibodies against it. However, in addition to having these properties in common with polioviruses, it differs immunologically, has only a low level of pathogenicity for T.C., is more pathogenic for suckling mice and not at all for adult mice, and produces a myositis in the sucklings. In other words it is also a typical Cocksackie A virus.

The pathologic picture produced in monkeys by the Cocksackie A7 strains studied, both Russian and American, could not be distinguished from that caused by poliovirus in 4 of the 7 animals examined. The remaining 3 presented lesions with a distribution differentiating their infection from that caused by poliovirus.

The findings of the Russian virologists, confirmed here, add one more facet to the

broad spectrum represented by the Cocksackie, poliomyelitis, and certain Echo viruses where the properties of one group blend sometimes rather extensively with those of the others, and all seem capable of causing related and sometimes identical clinical and histological manifestations in experimental animals and man. Certainly our present knowledge of these relationships should emphasize the necessity of search for these other viruses in paralytic cases where the specific etiology of poliovirus cannot be established. It must be remembered, however, that in the case of Cocksackie A7, at least, isolation can be accomplished more easily by inoculation of suckling mice or even monkeys than by currently available T.C. methods. Furthermore, the results reported here make it desirable that more extensive studies than those thus far reported(17) be carried out with Cocksackie viruses in monkeys or chimpanzees to determine if these characteristics may be found in types other than A7.

Conclusions. 1. The Russian Type 4 poliovirus is Cocksackie A7. 2. The Russian virus is capable of causing polio-type lesions in the monkey CNS. 3. American strains of Cocksackie A7 are likewise pathogenic for monkeys and in certain inoculated animals produce histological changes in the CNS which are indistinguishable from those caused by polioviruses. 4. Cocksackie A7 infections are probably prevalent in the U.S.

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A Proposed Potency Test for Smallpox Vaccine. I. Evaluation of Chick Embryo LD₅₀ Method. (23305)

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The official potency requirement in force today, at least in the United States, for release of smallpox vaccine is based on a modification of the method of Calmette and Guérin(1,2), suggested by Force and Leake (3). This method has been most valuable over the years, but those engaged in the production and distribution of smallpox vaccine are familiar with the irregularities to which the rabbit test is subject and are aware of the lack of quantitative criteria for interpretation and recording of results. In fact, only relative values of the potency of a vaccine under test can be estimated, since a control vaccine of acceptable potency always has to be titrated in parallel on the same animal. The desirability, therefore, of a test method less susceptible to variation and capable of measuring the absolute titer of smallpox vaccine is quite evident. A titration method for vaccinia virus based on the number of pocks formed on the chorioallantoic membrane of embryonated hen eggs was first described by Burnet(4). Although this technic was found most effective for titration of vaccinia virus preparations of relative purity, it has not generally proved very satisfactory with the comparatively coarse smallpox vaccine suspensions. Furthermore, even with purified vaccinia virus, difficulties have been encountered with this method unless applied under speci-

fied optimal conditions(5).

Observations in this laboratory over the years(6) have indicated that embryos inoculated on the chorioallantoic membrane with dilutions of smallpox vaccine eventually die from the infection, and that death is invariably associated with the presence of pocks on that membrane. Moreover, there seems to be a linear relation between log-dilution of vaccine and reciprocal embryo survival time, similar to that reported for the Psittacosis-LGV group of viruses(7,8).

The present communication reports results of repeated titrations of the same smallpox vaccine preparation by the chick embryo LD₅₀ method over a period of two years.

Materials and methods. Virus strain. The virus used in this study originated from human vaccination scabs obtained from the New York City Board of Health in 1909. It has since been maintained in this laboratory by irregular, alternating passages through calves, rabbits and humans, and has been used continuously for commercial production of calf lymph vaccine by the standard procedure. *Smallpox vaccine.* All titrations to be reported here were carried out with the same *glycerinated control vaccine virus* prepared in accordance with the minimum requirements set forth by the National Institutes of Health, Bethesda, Md., and meeting the requirement

of potency on humans and rabbits(9). It consisted of a 20% calf lymph suspension in 45-50% neutral glycerine. At the onset of this study, the vaccine was distributed in 1.0 ml amounts in sealed glass ampoules and stored at a constant -60°C . Individual ampoules were thawed out at the time of use. *Chick embryo and inoculation methods.* The chick embryos employed were of the Leghorn breed. They were routinely received from the same supplier on the day of inoculation, when 11 to 12 days of age. Regardless of the route of injection, a uniform inoculum volume of 0.25 ml was used per embryo. *Allantoic cavity (AC) inoculation.* The margin of the air sac was located by candling, and a small hole was drilled through the shell at a point $\frac{1}{4}$ " above the margin and as far as possible from the embryo. Inoculation was done by inserting a $\frac{3}{4}$ "-23 gauge needle full length through the hole at a slight angle toward the shell and with the egg standing upright on its small end. *Yolk sac (YS) inoculation.* Provided the air sac was in its proper position, a small hole was drilled at the highest point of the large end of the shell. A 1"-21 gauge needle, inserted full length vertically through the hole as the egg stood upright, was used for inoculation. *Chorioallantoic membrane (CAM) inoculation.* In a departure from the classical method of Beveridge and Burnet (10), routine CAM inoculations were carried out on large numbers of embryos as follows: The embryo was held against the candler in a horizontal position with an appropriate area of the CAM uppermost, and two $1/16$ " holes were made with an electric hand drill, one at the center of the natural air sac and the other over the area to be inoculated. Care was always exercised not to injure the underlying shell-membrane (SM). At first, a sterile hypodermic needle was used to pierce the SM over the air sac and then, bevel up, to nick it very lightly over the CAM. Experience soon indicated that the CAM separated from the SM and collapsed just by gravitation, expelling the air from the natural air sac. Only occasionally was it found necessary to apply a slight suction over the air sac while nicking the SM over the CAM. Following inoculation by any of these 3 routes, the shell holes

were sealed with collodion and the embryos incubated at 36° - 37°C . Care was taken to place the CAM inoculated embryos in a horizontal position with the collodion seal uppermost. *Vaccine titration and interpretation of results.* The virus was diluted routinely in 10-fold steps in beef heart infusion broth containing 500 units of penicillin and $100\text{ }\mu\text{g}$ of streptomycin per ml. Inoculated eggs were candled every day at the same time, and dead embryos were removed and examined carefully. Those dead 24 hours after inoculation were excluded from calculation, as were those who died after 48 hours without showing evidence of infection. Similarly, embryos inoculated on the CAM, whose death on any day could not be associated with virus multiplication by the presence of pocks, were considered to have died from other causes and were also excluded. Calculation of LD_{50} titers was done by the Reed and Muench formula(11) at the end of various incubation periods.

Results. Comparative titration of smallpox vaccine by CAM, AC and YS routes. It has been indicated(6) that, whereas a $10^{-5.0}$ dilution of the control vaccine virus caused death of the embryos in 96 to 120 hours when inoculated on the CAM, a $10^{-3.0}$ dilution of the same vaccine was required by the AC route to kill the embryo within the same interval. Variation in susceptibility of embryos depending upon route of inoculation was repeatedly observed in subsequent experiments as illustrated by the following typical simultaneous titration: the control vaccine virus was diluted from $10^{-3.0}$ to $10^{-9.0}$, and each dilution was inoculated into 6 embryos by either the CAM, AC, or YS routes. The time of death at each dilution and the LD_{50} endpoint for each route of inoculation on the 7th day are given in Table I. It is readily seen that the LD_{50} titer is at least 2 logs higher in the CAM group than in the groups inoculated by either the AC or YS routes. Because of the greater susceptibility of embryos injected on the CAM, this route was employed exclusively for all subsequent titrations.

Repeated titrations of smallpox vaccine by the CAM route. Between Sept. 1949 and

TABLE I. Comparative Chick Embryo Titration of Smallpox Vaccine by Various Routes of Inoculation.

Route of inoc.	Virus dilution	Death pattern: No. dead/No. inoc. (hours)							Accumulation		
		24	48	72	96	120	144	168	Dead	Alive	LD ₅₀ titer
CAM	10 ⁻³	0/6	3/6	6/6					31	0	
	-4		0/6	5/6	6/6				25	0	
	-5		"	3/6	"				19	0	
	-6	1/5*		0/5	5/5				13	0	
	-7				0/6	4/6	4/6	5/6	8	1	10 ^{-7.8}
	-8	" *					0/5	2/5	3	4	
	-9				"	1/6†	0/5	1/5	1	8	
	10 ⁻⁸	0/6	2/6*	0/4	0/4	4/4			14	0	
	-4				0/6	2/6	2/6	5/6	10	1	
AC	-5		1/6*				0/5	4/5	5	2	10 ^{-5.4}
	-6						0/6	1/6	1	7	
	-7		1/5*					0/5	0	12	
	-8							0/6	0	18	
	-9							"	0	24	
	10 ⁻⁸			1/6	1/6	6/6			17	0	
YS	-4				0/6	3/6	3/6	6/6	11	0	
	-5						0/6	3/6	5	3	10 ^{-5.8}
	-6		1/6*				0/5	2/5	2	6	
	-7							0/6	0	12	
	-8							"	0	18	
	-9		" *					0/5	0	23	

* Traumatic deaths: excluded from calculation.

† No pocks on CAM: " " " "

Sept. 1951, the same control vaccine virus was titrated on the CAM 28 times using dilutions ranging from 10^{-6.0} to 10^{-9.0} and 6 embryos per dilution. In an effort to determine the optimal interval, LD₅₀ titers were calculated at the end of 5, 6, 7, and 8 days incubation. Geometric means of the 28 titrations at these time intervals, and the standard deviation of log titers are shown in Table II. Although the majority of deaths occurred by

TABLE II. Geometric Means of Chick Embryo LD₅₀ Titers of Control Vaccine Virus Incubated for 5, 6, 7 and 8 Days Post-Infection.

Days incubation	No. of tests	Mean LD ₅₀ titers	Stand. dev. of log titers
5	27	10 ^{-6.6}	.43
6	28	10 ^{-7.3}	.34
7	28	10 ^{-7.5}	.33
8	25	10 ^{-7.6}	.36

the 5th day, the number of embryos dying was significant until the 7th day, after which relatively few died. The standard deviation of log titers was lowest on either the 6th or 7th day. An incubation period of 7 days was, therefore, selected for termination of titrations, and the frequency distribution of LD₅₀ titers from the 28 titrations, given in Table III, shows that titers lower than 10^{-7.2} or higher than 10^{-8.0} were only infrequently obtained.

These titrations, made at regular intervals over a period of 2 years, afforded a unique opportunity to determine whether seasonal changes had any effect on susceptibility of the embryo to vaccinia virus. The 7-day data were, therefore, reexamined according to the seasonal distribution of the titrations (Table IV). Because of the small number of tests,

TABLE III. Frequency Distribution of Chick Embryo LD₅₀ Titers of Smallpox Vaccine on 7th Day of Incubation.

LD ₅₀ titers	10 ^{-6.7}	10 ^{-7.0}	10 ^{-7.2}	10 ^{-7.4}	10 ^{-7.5}	10 ^{-7.6}	10 ^{-7.7}	10 ^{-8.0}	10 ^{-8.4}	Total	Geometric mean	Stand. dev. of log titer
No. of assays	1	1	4	5	7	4	2	3	1	28	10 ^{-7.5}	.33

TABLE IV. Seasonal Variation of Mean Chick-Embryo LD₅₀ Titers of Smallpox Vaccine on 7th Day of Incubation.

Season	No. of titrations	Mean LD ₅₀ titers	Stand. dev. of log titers
Spring (Mar. 23-June 15)	9	10 ^{-7.4}	.36
Summer (June 22-Sept. 8)	8	10 ^{-7.7}	.39
Fall & Winter (Sept. 28-Mar. 16)	11	10 ^{-7.4}	.20
Total	28	10 ^{-7.6}	.33

those made in fall and winter were calculated together. It can be seen that seasonal variations of mean titers and of respective standard deviations were not significant.

Discussion. The chick embryo LD₅₀ method, using 24 embryos, 6 at each of 4 dilutions, was used in this study. Applying Gaddum's formula(12) to determine standard error of a log LD₅₀ calculated by the Reed and Muench method, a theoretical value of 0.30 in logarithmic units was obtained. The 28 titrations gave individual log LD₅₀ titers ranging from -6.7 to -8.4 with a standard deviation of 0.33. This is in good agreement with the theoretical value of 0.30, and accordingly, there seems to be no indication that the LD₅₀ titer may change appreciably with time. It would thus be legitimate to calculate potency of a sample in relation to long-term average LD₅₀ titer of the control vaccine, rather than to titrate each new vaccine lot in parallel with the control vaccine, as is done in the rabbit test. Check titrations of the control vaccine in the chick embryo, at periodic intervals, however, may be advisable.

These results would seem to indicate that production lots of vaccine should have minimum chick embryo LD₅₀ titers of 10^{-7.5±0.33} to be of acceptable potency. From the frequency distribution of 7-day titers of the standard vaccine, it seems reasonable to assume that, should a vaccine lot fail to meet the lower limit on first test, one, or at most 2 additional titrations may be required to determine definitely whether it should be retained or rejected. The chance for such an occurrence, however, is of the order of 2 in 28. Since this study was undertaken, many

production lots of calf lymph prepared in these laboratories, as well as several vaccines of chick embryo origin, were tested for potency by both the conventional rabbit and the chick embryo LD₅₀ method. In not a single instance did a vaccine which passed the rabbit test fail to reach a chick embryo LD₅₀ titer as described above. Admittedly, the chick embryo LD₅₀ titer would assume a greater degree of significance if it were correlated with human vaccination by simultaneous titration of the same vaccine in humans and in the chick embryo. Such a study is under consideration.

Summary. 1) A glycerinated control smallpox vaccine of calf lymph origin was titrated for a total of 28 times, over a period of 2 years, by the chick embryo LD₅₀ method. Calculations of LD₅₀ titers were made at the end of 5, 6, 7, and 8 days incubation, and those of the 7th day were found to be optimal. Individual titers on the 7th day ranged from 10^{-6.7} to 10^{-8.4}, giving a geometric average titer of 10^{-7.5} for the control vaccine with a standard deviation of 0.33 log unit. No significant seasonal variations of the average LD₅₀ titers were noted. 2) The precision of the method and its applicability to the potency testing of smallpox vaccine have been discussed.

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New Antigenic Variant in Far East Influenza Epidemic, 1957. (23306)

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During the late spring of 1957, epidemic influenza characterized by clinically mild illness and by high attack rates occurred throughout the Far East, following a winter of relatively low influenza prevalence throughout most of the world. The viruses responsible for the Far East epidemic were shown(1) in this laboratory to be type A influenza of strikingly different antigenic composition from strains recovered in previous years. This paper summarizes the clinical and epidemiological reports from the Far East outbreak, presents the results of the detailed antigenic analyses of these viruses and the antibody response to them in patients, and gives information concerning the biological properties of these viruses and the antibody status of U.S. military personnel.

Materials and methods. Far East virus strains designated A-Japan-305-57 and A-Japan-307-57 were recovered in embryonated eggs from American military personnel by Drs. S. E. Grossberg and I. Gresser at U.S. Army 406th Medical General Laboratory, Zama, Japan, and were forwarded to this laboratory. The A-Malaya-309-57, 310-57, and 311-57 strains were recovered in Malaya by Dr. J. H. Hale of the University of Singapore. A-Hong Kong-304-57 virus was isolated in this laboratory from throat washings collected by Dr. C. Kraul of the 406th Medical General Laboratory from a patient in the Hong Kong outbreak. The A-Formosa-313-57 strain, recovered at the Taiwan Serum Vaccine Laboratory, was sent by the U.S. Naval Medical Research Unit #2 to the 406th Medical General Laboratory and forwarded

to this institution. **Prototype strains.** Strains selected from previous epidemics for purpose of comparison are referred to as prototype strains. The prototype virus designated A-Japan-301-56 was recovered in Dec. 1956 in Japan and was furnished by Dr. M. Kitaoka, of the Nat. Inst. of Health, Tokyo, Japan. Miss Betty Ransom of the U.S. Army Hawaiian Med. Laboratory furnished the A-Hawaii-303-56 strain. The remaining prototype viruses used to prepare antigens or antisera in this laboratory have been described (2,3). **Hemagglutinating antigens** were allantoic fluids from embryonated eggs infected with influenza virus. The antigens prepared from the new Far East isolates were in egg passage 2 to 5 and titered 1:40 to 1:320 in tests with human "O" red blood cells. Drs. Thomas Francis, Jr., Keith Jensen, Bernice Eddy, or Robert M. Chanock furnished hemagglutinating antigens for the A-AA-1-57 (Mich.), A-Denver-2-57 (Colo.), A-AA-4-56 (Mich.), A-Ned-36-56 (Netherlands), A-Sendai-5-56 (Japan), B-GL-1739-54 (Ill.), A-Swine, and type D-Sendai (Japan)(4,5) viruses. The remaining antigens were standard reagents used by this laboratory and the strains employed in their preparation are described above or elsewhere(2,3). The methods used to prepare the antigens have been described(2,3,6). **Animal antisera.** Sera against the new Far East isolates were prepared in chickens by methods described earlier(2,3,6). Each chicken received 20 ml of undiluted infected allantoic fluid on 2 successive days and the animal was bled 9 or 10 days following the first injection. The ferret

antisera were obtained from Dr. Fred Davenport and the chicken A-Swine antiserum was furnished by Dr. B. Eddy. Drs. K. Jensen and R. M. Chanock supplied the B-GL-1739-54 and D-Sendai antisera. The Newcastle Disease Virus antiserum was obtained from Miss Audrey Somer of the U.S. Dept. of Agriculture. The remaining sera were standard reagents used in this laboratory and the strains of virus employed in their preparation are described above or earlier(2,3). *Complement-fixing antigens.* Embryonated eggs, incubated for 9 days, were inoculated into the allantoic cavity with 0.2 ml of infected allantoic fluid diluted $10^{-2.0}$. The chorioallantoic membranes, harvested after 36 hours' further incubation, were washed, triturated with 1 ml of physiological saline/g weight of membrane, then frozen and thawed 3 times. The supernate obtained following centrifugation at 15,000 rpm in the angle centrifuge for 30 minutes was the antigen and this usually titered 1:64 or 1:128 when tested with 2 units of human influenza A or B convalescent serum antibody by the complement-fixation (CF) technic. *Paired sera from influenza patients.* Acute and convalescent serum specimens were collected by Dr. C. Kraul from both Oriental and Caucasian patients in Hong Kong during late April. The paired sera from U.S. military patients aboard a ship at Yokosuka, Japan, were drawn by Dr. S. E. Grossberg of the U.S. Army 406th Med. General Laboratory. The paired sera from cases of influenza in the outbreak in the Philippines were forwarded by the 406th Med. Gen. Laboratory. The remaining paired sera were from patients with influenza A or B in outbreaks which occurred in military personnel in Hawaii, Md., Kan., D.C., or Va. during the years 1955 (influenza B), 1956, or 1957 (influenza A). *Serological procedures.* The hemagglutination and hemagglutination-inhibition titrations were performed using human "O" cells according to the method of the Committee on Standard Serological Procedures in Influenza Diagnostic Studies(7). Treatment of sera with cholera filtrate to remove non-specific inhibitors was carried out according to Mulder *et al.*(8). The complement-fixation test procedure has been de-

scribed(9). Primary incubation in the CF tests was carried out at 37°C for 75 minutes. All virus and serum titers are expressed in this report as the initial dilution prior to addition of the other reagents.

Background epidemiological information. Winter of 1956-57 prior to Far East epidemic. According to present information(1,10-12), the first occurrence of influenza for the 1956-57 respiratory disease season was during Nov. and Dec., 1956, in Japan. The attack rates were high in certain outbreaks, especially in children, necessitating the closing of schools. The disease occurred also in the U.S. Armed Forces in Japan. In U.S.A., the disease was first noted at Great Lakes Naval Training Center, Ill. during Dec., 1956 and by Apr. 1957, localized and scattered outbreaks had occurred throughout much of the nation. There was comparatively little influenza in France, England or Wales during the same period. However, epidemic influenza was widespread throughout Central Europe and the Scandinavian countries during March and April. Clinically, the illness in these outbreaks was mild and of 3 to 4 days duration. The attack rates were as high as 15% in certain "closed" populations such as schools or other institutions. These epidemics were caused, predominantly, by type A prime influenza strains which resembled closely the 1956 Dutch prototype strain A-Ned-36-56. Influenza B and C were also reported but the incidence was low. *Far East outbreak, Spring 1957*(1,10-12). The first official report of influenza in the Far East during the spring of 1957 described an epidemic in Hong Kong which began early in April and affected both Oriental and Caucasian populations. The epidemic was reported in Formosa by the end of April and in early May in the Philippines and Malaya. Late in May the epidemic spread to India, South Viet Nam and Japan. Epidemics occurred also aboard many U.S. Navy vessels which had contact with the disease in Far East ports during that period. Unconfirmed reports indicate that influenza epidemics may have been widespread in China prior to the Hong Kong epidemic. Prominent features in the epidemics have been the rapid spread of the disease and the

TABLE I. Hemagglutination-Inhibition and Complement-Fixation Titers of Paired Sera from Cases of Influenza in Far East and in Recent Outbreaks (1955-57) in Tests with Far East and Prototype Influenza Viruses.

Cases tested			Serum titers obtained with antigens									
			Complement-fixation ("soluble" antigen)				Hemagglutination-inhibition* (viral antigen)					
			Far East viruses		Prototype viruses		Far East viruses			Prototype viruses		
			A-Japan 305-57	A-Japan 307-57	A-Hawaii 303-56	B-Va, 301-55	A-Japan 307-57	A-Hong Kong 304-57	A-Malaya 309-57	A-Denver 2-57	A-Japan 301-56	A-FLW, 1-52 B-IB1 (1950)
Outbreak	Pt.	Serum sample										
<i>Far East 1957</i>												
Hong Kong	1	A	5	5	5	<5	<5	<5	<5	<5	5	20
		C	40	40	80	"	40	40	40	10	10	40
	2	A	<5	<5	<5	"	<5	<5	<5	<5	10	20
		C	40	40	80	"	40	20	20	5	"	"
	3	A	<5	<5	<5	"	<5	<5	<5	<5	"	"
		C	40	40	80	"	20	10	20	5	"	"
	4	A	<5	<5	5	5	<5	<5	<5	<5	<5	40
		C	80	80	80	5	20	10	10	"	5	"
Japan (ship)	5	A					<10			20	20	20
		C					40			"	"	40
	6	A					<10			5	"	80
		C					20			10	"	"
	7	A					<10			<5	5	20
		C					"			20	20	160
<i>U. S. A.</i>												
Influenza A (1956-57)	8	A	5		<5	<5	<5	5	<5	5	20	40
		C	80		80	"	10	20	10	20	320	320
	9	A	<5		<5	"	<5	<5	<5	<5	10	40
		C	320		320	5	10	"	10	80	160	640
	10	A	<5		<5	<5	<5	"	<5	<5	<5	20
		C	20		20	"	"	"	"	10	10	160
	11	A	<5		<5	"	"	"	"	<5	5	20
		C	20		40	"	"	"	"	10	10	320
	12	A					"	"	"	<5	5	20
		C					"	"	"	40	40	160
	13	A	<5		<5	"	"	"	"			20
		C	"		"	160	5	"	5			320
Influenza B (1955)	14	A	"		"	<5	<5	"	<5			80
		C	5		5	80	"	"	"			40

* All sera were treated with cholera filtrate to remove non-specific inhibitor.

high attack rates, varying from 10-30% in both oriental population and Armed Forces. The illness occurred in persons of all ages and was generally mild lasting 3 to 4 days frequently accompanied by fever up to 103°F. Deaths attributable to influenza have been infrequent except in the Philippines where up to 200 children are said to have died as a result of influenza and in India where 21 deaths have been reported to date. The viruses recovered from the recent Far East outbreaks

are, as shown in this report, of A type and are strikingly different antigenically from those which were prevalent during the winter of 1956-57 or in preceding years.

Results. One outstanding property of influenza A virus is the change in antigenic constitution over succeeding years(2,3,13-19). Hence, in the detailed analyses reported here, prototype viruses were employed(2,3) which cover the antigenic spectrum of known influenza viruses and represent the various eras

TABLE II. Comparison of Far East Influenza A Isolates with Prototype Strains in Hemagglutination-Inhibition Tests with Chicken Antisera.

Hemagglutination-inhibition titers obtained in tests with antigens:																		
	Far East viruses				Prototype viruses							Other prototype viruses (homo- logous titers)						
	A-Japan 305-57	A-Japan 307-57	A-Hong Kong 304-57	A-Malaya 309-57	A-Malaya 310-57	A-Malaya 311-57	A-AA, 1-57	A-Denver 2-57	A-Japan 301-56	A-Hawaii 303-56	A-AA, 4-56		A-FLW, 1-52	A-FW, 1-50	A-FM1 (1947)	A-PR8 (1934)	A-WS (1933)	A-Swine 301-55
Chicken antisera against viruses: (type and strain)																		
<i>Far East viruses</i>																		
A-Japan-305-57																		
#9488	100	400	400	400	400	200	<25	<25	<50	<50	<25	<25	<25	<25	<25	<25	<25	<25
#B891	200	"	"	800	"	400	"	"	"	"	"	"	"	"	"	"	"	"
A-Malaya-311-57	100	800	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
<i>Prototype viruses</i>																		
A-Japan-301-56 (Dec.)	<50	<25	<50	<25	<25	<50	<25	<25	400	400	<25	<25	<25	<25	<25	<25	<25	<25
-Hawaii-303-56	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
-AA-4-56	"	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
-FLW-1-52	"	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
-FW-1-50	"	"	"	"	"	"	"	"	"	800	<25	<25	<25	<25	<25	<25	<25	<25
-FM1 (1947)	<50	"	"	<50	"	"	<25	<25	"	800	<25	<25	<25	<25	<25	<25	<25	<25
-PR8 (1934)	"	<50	"	"	"	"	"	"	"	1600	<25	<25	<25	<25	<25	<25	<25	<25
-WS (1933)	"	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
-Swine	"	<50	"	"	"	"	"	"	"	3200	<25	<25	<25	<25	<25	<25	<25	<25
B-Va-301-55	"	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
-GL-1739-54	"	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
-IB1 (1950)	<25	<25	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
-Lee (1940)	<50	<50	"	"	"	"	"	"	"	1600	<25	<25	<25	<25	<25	<25	<25	<25
C-1233 (1947)	<25	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
D-Sendai	<50	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
NDV	"	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25
Mumps	"	"	"	"	"	"	"	"	"	400	<25	<25	<25	<25	<25	<25	<25	<25

All sera were treated with cholera filtrate to remove non-specific inhibitor. Specific antibody titers in the range of 1:400 to 1:1600 were obtained when the untreated Far East sera were tested with Far East viruses.

of their prominence.

Serological tests with paired sera from cases. Table I presents the CF and hemagglutination-inhibition test results with paired sera from cases of influenza among Orientals and Caucasians in the Far East outbreak and from patients in previous outbreaks in the U.S.A. employing, as antigens, Far East viruses and previous prototype strains. The cases in the Far East were clearly established as influenza A by demonstration of significant (4-fold or greater) CF antibody titer increases in paired sera employing CF antigen prepared from prototype A-Hawaii-303-56 virus and by demonstration of significant increases in titer in patients with influenza A in previous outbreaks using, as antigen, Far East viruses. Thus, there was no demonstrable difference between the Far East and previous A viruses insofar as reaction with the "soluble" CF antigen components of the viruses were concerned. This was not the case with the hemagglutinating antigens which measure antibody to the virus itself. In the Far East cases, antibody response was almost exclusively against the Far East viruses and, conversely, the responses in cases from previous epidemics were nearly all against the previous prototypes. In 3 instances, Far East case 1 and USA cases 8 and 9, significant titer increases were noted against both kinds of virus; the rise against the homologous agent was the greatest in each instance. Far East case 7 was unique in that the patient's sera showed an 8-fold titer increase against prototype FLW-1-52 but nothing against Far East

virus. It seems most likely, in this instance, that the patient was infected with a 1956 prototype during the epidemic although Far East virus infection cannot be excluded. Far East virus appeared responsible also for the epidemic in the Philippines. Five of 7 paired sera from cases in the Philippines, not included in the table, showed diagnostic titer rises when tested with Far East A-Japan-307-57 virus but not with prototype A-Japan-301-56 virus. There was no evidence of influenza B in the epidemic in the Far East.

Antigenic analysis of Far East viruses with chicken and ferret antisera. Table II summarizes the results of cross-hemagglutination-inhibition tests with chicken antisera between the Far East agents and prototype viruses listed. No demonstrable antigenic relationship was shown in tests between Far East viruses and influenza A prototypes from early 1957 through 1933 and including swine virus. The A-Formosa-313-57 virus, not included in the Table, gave results similar to the other Far East strains. Likewise, there was no antigenic relationship to influenza B, C, D or NDV or mumps viruses. The striking antigenic difference between Far East and 1956-57 influenza A strains was shown further in tests (Table III) with ferret antisera against 1956 and early 1957 influenza A strains recovered in the USA, Europe and Japan. The old 1956-57 prototypes were a remarkably homogeneous group except for minor variation exhibited by strain A-Sendai-5-56. None of these sera showed any detectable reaction with the new Far East agents.

TABLE III. Comparison of Far East Influenza A Isolates with Recent (1956-57) A Type Viruses in Tests with Ferret Antisera.

Ferret antisera* against recent A viruses: (type and strain)	Hemagglutination-inhibition titers obtained in tests with antigens									
	Far East viruses					Recent (1956-57) A type viruses				
	A-Japan 305-57	A-Japan 307-57	A-Hong Kong 304-57	A-Malaya 309-57	A-Malaya 310-57	A-Denver-2-57	A-AA-1-57	A-AA-4-56	A-Ned-36-56	A-Sendai-5-56
A-Denver-2-57	<20	<20	<20	<20	<20	640	2560	80	1280	<20
-AA-1-57	"	"	"	"	"	160	640	20	320	"
-AA-4-56	"	"	"	"	"	80	160	320	20	20
-Ned-36-56	"	"	"	"	"	160	640	40	320	<20
-Sendai-5-56†	"	"	"	"	"	20	80	80	<20	160
										640
										1280
										40
										320
										80

* All ferret antisera were treated with cholera filtrate to remove non-specific inhibitor.

† Not to be confused with type D Sendai virus.

TABLE IV. Antibody Status, American Military Cadre Personnel for Far East Influenza Viruses and for Prototype Strains.

Serum* titer	Hemagglutination-inhibition titers						
	Far East viruses				Prototype strains		
	A-Japan 305-57	A-Japan 307-57	A-Malaya 309-57	A-Hong Kong 304-57	A-Japan 301-56	A-FLW 1-52	B-IB1 (1950)
<1:5	30	27	24	29	6	2	0
1:5	0	2	4	1	6	2	1
1:10	0	1	2	0	8	7	4
1:20	0	0	0	0	6	5	12
1:40	0	0	0	0	3	8	9
1:80	0	0	0	0	0	4	3
1:160	0	0	0	0	0	1	1
1:320	0	0	0	0	1	0	0
1:640	0	0	0	0	0	1	0

* All sera treated with cholera filtrate to remove non-specific inhibitor.

Antibody status of selected military personnel. Distribution of hemagglutination-inhibiting antibody against 4 Far East viruses, compared with 3 prototype strains, in tests of 30 sera from enlisted cadre personnel, is shown in Table IV. These persons, in keeping with regulations requiring influenza vaccination in the military, had been vaccinated prior to beginning of 1957. The high levels of antibody against the old prototype strains A-Japan-301-57, A-FLW-1-52 and B-IB1 were as expected. However, there was a striking deficiency in antibody against the Far East isolates in these persons. None showed detectable antibody against A-Japan-305-57 virus and 6 showed inhibition against the other Far East strains in very low titers, *i.e.*, 1:5 or 1:10.

Biological characteristics of the Far East viruses. The Far East viruses were readily recovered from patients' throat washings by passage in embryonated eggs inoculated into the amniotic cavity. Following initial recovery, the viruses grew readily in the allantoic cavity giving hemagglutination titers of 1:80 to 1:320 when tested with human "O" or with chicken erythrocytes and incubated at room temperature or at 4°C. Early small volume commercial pools of allantoic fluid harvested from embryonated eggs infected with the A-Japan-305-57 virus gave low titers in the range of 16 to 92 chicken cell agglutination (CCA) units/ml as compared with 300 or more units expected when strains well adapted to growth in eggs are employed. The Far East viruses showed the usual hemag-

glutination pattern and did not elute more rapidly from red blood cells than did the prototype A viruses. All Far East agents studied, except A-Japan-305-57 and possibly A-Formosa-313-57, proved highly susceptible to non-specific inhibitors in human and ferret sera. This inhibitor was destroyed readily by cholera filtrate. Strains A-Japan-305-57 and A-Formosa-313-57, only, were suitable for diagnostic purpose with untreated sera. Certain of the early passage egg fluids of strain A-Japan-305-57 were not readily inhibited by homologous immune serum and appeared to be in Q phase(13). A-Japan-305-57 virus, purified from infected allantoic fluid and examined in the electron microscope, was shown by Dr. R. E. Hartman of this laboratory to consist primarily of virus in filamentous form as is characteristic for newly recovered type A strains(20). This strain of virus was not mouse virulent and induced only minimal lung lesions on primary and second passage in mice inoculated by the nasal route.

Discussion. Continuous change in antigenic composition over succeeding years is a prominent characteristic of influenza viruses (2,3,13-19). This may consist of sudden and marked change such as the shift from classical type A to A prime in 1946-47(21,22) or by more gradual and progressive alteration such as observed in the A prime group since 1947 (3,15,16,23,24). The extent of the antigenic difference between the Far East and the type A prototype viruses recovered in recent years cannot be defined precisely because the difference is so great as to obviate the demonstra-

tion of cross-reactions between these groups in the present tests with the available chicken and ferret antisera. Thus, any similarities which might be present are outside the range of measureability in the tests employed. It may be concluded, from our data, however, that modification of antigenic composition of the Far East viruses represents a greater change from the recent A prime era than occurred in the 1946-1947 shift from the type A to the type A prime viruses. This conclusion is supported by the following: First, an antigenic relationship between the type A and A prime viruses is readily demonstrable (2,13, 16,21) by the hemagglutination-inhibition method using chicken and ferret antisera while such relationship between the A prime and Far East viruses is not shown. Second, paired sera from type A prime cases of influenza which occurred in the 1947 outbreak (21,22) and in later epidemics, showed increases in titer against the old prototype A strains, including 1934 PR8, which were as great as or greater than the rises against the homologous A prime viruses. It cannot be concluded from these data, however, that the Far East viruses consist entirely of antigens created *de novo* with no relationship to viruses of the past. Evidence for some relationship is presented in the occasional small titer increases observed in the tests with paired sera from patients and virus strains from the Far East epidemic and from epidemics of previous years. Additionally, the Far East viruses share the same group-specific "soluble" CF antigen as all the type A viruses.

The outbreak in the Far East was characterized by sudden appearance in epidemic proportions of disease caused by a new or hitherto unrecognized form of influenza A virus which followed, within 4 months, the occurrence of epidemics of classical A prime influenza in Japan, the U.S.A. and parts of Europe. The high attack rates among Orientals and Caucasians, the apparent lack of protection (1) in Service Personnel given the standard vaccine (Formula, A-Swine, A-FM1, and B-GL-1739-54), and the deficiency of Far East virus antibody in selected "normal" persons and in patients recently recovered from influenza A prime infection, all indicate

a general low level of immunity against the new agents in the human population and the potential for spread of the virus throughout the U.S.A. and probably much of the world. The reports from the Far East and Asia have indicated a generally mild form of illness, except for deaths reported from the Philippines and India. Whether the Far East viruses will increase in virulence on further rapid passage in the susceptible population and whether the occurrence of the disease during the normal winter respiratory disease season will operate to enhance its severity are important observations for the future.

The epidemic occurrence in the Far East with the new virus serves to reemphasize the importance of the world-wide influenza surveillance carried out by the Armed Forces and by the World Health Organization (25) during the past decade. In the present instance, the virus was recovered and analyzed shortly following the first known epidemic occurrence and the characterization was accomplished soon enough to provide what may prove to be sufficient time for production of vaccine in advance of the next expected influenza season in the U.S.A.

Based on past history, it is not unreasonable to expect that epidemics caused by the Far East virus will occur in the Southern hemisphere during the Northern summer of 1957 and that epidemic prevalence will be experienced in the Northern hemisphere during the late Fall and Winter of 1957-58. In view of the ample time for widespread dissemination of the Far East agent, outbreaks of multicentric origin as well as by contiguous spread may be anticipated. It seems likely also that the A prime virus will not be displaced immediately and that it will occur also during the next epidemic season.

Summary. Viruses responsible for the widespread epidemic of influenza in the Far East during the spring of 1957 were caused by a type A virus which represented a major shift, antigenically, from the type A, A prime and swine influenza viruses of previous years. These conclusions are based on findings in tests of Far East and prototype A viruses with chicken and ferret antisera against Far East and prototype strains and in tests with

paired sera from patients in the Far East and previous epidemics. The marked antigenic shift in Far East viruses, the deficiency of antibody against them in the human population tested, the rapid spread of the disease in the Far East, and the relatively high attack rates, all indicate the potential importance of the new viruses as a health problem in the U.S.A. and probably much of the world. During the present period, the new viruses are being called Far East influenza virus, 1957.*

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* Since the present manuscript was written Dr. Christopher Andrewes, in a personal communication, has also suggested this interim name for the Far East viruses.

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